C2 530/1009 (ATCC VR 2451), cpts RSV 530/1030 (ATCC VR 2455), RSV B-1 cp52/2B5 (ATCC VR 2542), and RSV B-1 cp-23 (ATCC VR 2579).

C3 114. (Amended) The recombinant RSV of claim 63 which is a complete virus.

C4 116. (Amended) The recombinant RSV of claim 63, formulated in a dose of 10^3 to 10^6 PFU of attenuated virus.

REMARKS

Claims 63-76 and 90-145 are pending in the application and reproduced in current form in the Appendix below. Claims 77-89 and 146-147 are withdrawn from consideration as drawn to a nonelected invention. Claims 64-76, 91, 92, 95-99, 102, 104-113, 125-126, 128, 130, 132, and 134-145 are withdrawn from consideration as directed to nonelected species. Claims 63, 90, 93-94, 100-101, 103, 114-124, 127, 131, and 133 are under examination to the extent that they read on the elected species as noted in the record. By this submission, claims 90, 101, 114, and 116 have been amended for clarity in accordance with the Office's suggestions. These amendments are fully supported by the disclosure and are intended for clarity and not to alter the scope of the subject claims. No new matter has been added to the application.

Information Disclosure Statement

The Office has acknowledged receipt and consideration of the Information Disclosure Statements filed on July 27, 2000 (Paper No. 4) and December 11, 2000 (Paper NO. 5).

Claim Objections

Claim 101 is objected to because of a typographical error in line 4. Appropriate correction is presented by amendment to the subject claim above.

Claim 114 is objected to under 37 CFR 1.75(c), as allegedly being of improper dependent form for failing to further limit the subject matter of a previous claim. In particular,

claim 114 originally recited the recombinant RSV(respiratory syncytial virus) of claim 63, "which is a virus." In accordance with the Office's suggestions, Applicants have amended the subject claim for clarity to place the claim in proper dependent form. In particular, amended claim 120 is now drawn to the RSV of claim 63, "which is a complete virus". As is clearly denoted in the specification and original claims, the "respiratory syncytial virus" of claim 63 is directed to an isolated infectious viral particle which comprises, at a minimum, the N, P, L and polymerase elongation factor proteins. As such, this basic virus particle is viable and infectious without the inclusion of non-essential components of a "complete" RSV. In contrast, as is also disclosed in the specification, it is within the scope of the invention to provide recombinant RSV that comprise essentially complete viruses, i.e., with all essential viral components and further including non-essential components as found in a complete, e.g., wild-type, RSV. Clearly representative of these teachings, the specification teaches that a number of non-essential genes, for example the SH, NS1 and NS2 genes, "can be ablated or otherwise modified to yield desired effects on virulence, pathogenesis, immunogenicity and other phenotypic characters. For example, ablation by deletion of a non-essential gene such as SH results in enhanced viral growth in culture." (see, e.g., page 38, lines 13-19).

In view of the foregoing remarks, the objection to claim 114 is believed to be obviated.

Patentability Under 35 USC § 112

Claims 90 and 115-116 are rejected under 35 U.S.C. 112, second paragraph, as allegedly being indefinite. In particular, the Office objects to the recitation of "a further modification" in line 1 of the subject claim as lacking strict antecedent basis in claim 63. Appropriate correction is presented by amendment to the subject claim above.

Claim 115 is objected to for reciting "a subviral particle", where the Office states that the term "virus" denotes a complete virion. On this basis it is asserted that the claim is indefinite, because it is allegedly "unclear how a complete virion or virus can also be a subviral particle." Applicants respectfully traverse this stated ground of rejection and submit that the subject claim fully and clearly conveys the subject matter of the invention to the skilled artisan. As noted above, the disclosure specifies that the "respiratory syncytial virus" of base claim 63 is

directed to an isolated infectious virus which comprises, at a minimum, the N, P, L and polymerase elongation factor proteins. This infectious viral unit is understood to comprise a basic virus particle that is viable and infectious--without the inclusion of non-essential components of a "complete" RSV. Applicants also clearly disclose in their specification that the invention includes recombinant RSVs that comprise essentially complete viruses, i.e., with all essential viral components and further including non-essential components as found in a complete, e.g., wild-type, RSV. In view of these teachings, the subject claim language is believed to fully convey the metes and bounds of the invention when considered in light of the specification. Withdrawal of the rejection of claim 115 is therefore respectfully requested.

Claim 116 is rejected on the basis that the recited dosage range is allegedly unclear. In particular, the Office suggests correcting the claim to include standard nomenclature for reciting plaque forming units, by writing the last numerical value as a superscript designating the number of log tens. Appropriate correction is is presented by amendment to the subject claim above.

Claim 101 is rejected under 35 U.S.C. 112, first paragraph for alleged lack of enablement. In particular, the Office states that it is not clear from the disclosure that the indicated ATCC deposits of biologically-derived mutant human RSV strains "meet all of the criteria set forth in MPEP 608/01 (p)(C), items 1-3." Applicants have attempted to identify the referenced rules in the cited passage of the MPEP, but could not ascertain from this passage clear directions for compliance. From the Office's suggestions, it is believed that submission of copies of the relevant Certifications of Deposit covering the indicated strains as described in the specification will likely complete the record. Accordingly, copies of Certifications of Deposit fully documenting the deposits of cpts RSV 248 (ATCC VR 2450), cpts RSV 248/404 (ATCC VR 2454), cpts RSV 248/955 (ATCC VR 2453), cpts RSV 530 (ATCC VR 2452), cpts RSV 530/1009 (ATCC VR 2451), cpts RSV 530/1030 (ATCC VR 2455), RSV B-1 cp52/2B5 (ATCC VR 2542), and RSV B-1 cp-23 (ATCC VR 2579), are forwarded herewith for consideration and formal entry in the record. Clarification is requested from the Office whether this submission brings the application into compliance, or whether a further submission (e.g., a Declaration) and/or amendment of the specification is required. If further compliance is deemed necessary,

the Office is respectfully urged to provide a clear citation of authority proscribing Applicants' requirements in this context.

Claims 117-124 are rejected under 35 U.S.C. 112, first paragraph, for alleged lack of enablement. The Office Action sets forth a number of general caveats that are asserted to render the invention impracticable without "undue experimentation." In particular, the Office contends that "[t]he art teaches that RSV vaccines comprising live attenuated virus often do not confer protection against subsequent RSV infection due to factors such as maternally acquired serum antibodies, incomplete immunity, and the existence of multiple antigenically diverse strains. (citing Murphy et al., Virus Res. 32:13-36, 1994, especially pages 14-15 and page 22, last partial paragraph, through page 26, first paragraph). In addition, the Office states that:

> The disclosure teaches how to make recombinant RSV, how to introduce a translation termination codon, and how to elicit an immunogenic response in BALB/c mice by administration of the mutant RSV. However, the disclosure does not teach that the immunogenic response is protective in humans against subsequent RSV infection in the presence of passively acquired maternal antibodies or that it is protective against subsequent infection with different strains of RSV. There are no working examples disclosing protection against subsequent infection with RSV or examples disclosing protection despite the presence of maternal antibodies.

Finally, the Office asserts that "[b]ecause the claims are drawn to vaccine compositions for protection against RSV in humans and because the art teaches that attenuated RSV vaccines are not protective against all subsequent RSV infections and are not protective in the presence of maternal antibodies, it would require undue experimentation by one of skill in the art to be able to practice the claimed invention."

Applicants respectfully traverse the foregoing grounds of rejection and submit that the teachings of the specification fully enable the artisan to practice the invention in a manner that is "reasonably commensurate" with the scope of the claims presented for review.

As an initial point in rebuttal to the Office's evidence and reasoning directed to the issues of enablement, Applicants respectfully submit that the appropriate standard for efficacy of a human vaccine does not require prevention of all subsequent infections by all

possible variants of a pathogen in all human populations. On the contrary, the enablement requirement of 35 U.S.C. § 112 only requires that there be a "reasonable correlation" between the disclosure and the scope of protection sought in the claims, having due regard for the nature of the invention and the state of the art. (See, e.g., In re Vaeck, 20 USPQ2d 1438 (Fed. Cir. 1991); Ex parte Jackson, 217 USPQ 804, 807 (Bd.Pat.App.Int. 1982); In re Fisher, 166 USPQ 18, 24 (CCPA 1970); MPEP § 706.03(n)). The proposed requirement for an optimal, comprehensively effective vaccine is also contrary to the central policy of U.S. patent law--to promote the useful arts. This policy is particularly permissive in the context of biomedical inventions such as human vaccines. As emphasized by the Board in Ex parte Aggarwal, 23 USPQ2d 1334 (Bd. Pat. Appl. Inter. 1992):

> Case law subsequent to Brenner is receptive to early filing of applications in the biomedical field . . .. (citing Brenner v. Manson, 383 U.S. 519 (1966)).

In the present field of invention, the artisan will not expect that useful vaccines are to be 100% effective in a single dose for all populations covering all potential variants of a targeted pathogen. On the contrary, the various obstacles to this goal proposed by the Office are anticipated, and accepted as "reasonable" and "ordinary" hurdles to the clinical refinement of all vaccine formulations and protocols. These refinement efforts do not rise to the level of "undue experimentation", particularly when the goal of the artisan is to implement a pioneering vaccine as provided by Applicants to ameliorate such a significant threat to human health as RSV.

Merely because the Office can point to certain obstacles that might render an embodiment of the claimed vaccine viruses inoperable in some instances--to comprehensively prevent all variants and manifestations of RSV--such obstacles do not support a finding of "undue experimentation." The Office rests its conclusion on certain passages of the Murphy et al. reference that discuss the complications of maternally acquired antibodies, incomplete immunity, and multiple, antigenically diverse RSV strains. A more in-depth analysis of this reference undermines the Office's conclusion of undue experimentation. In particular, the Murphy et al. reference addresses each of the obstacles advanced by the Office and provides solutions that parallel the teachings and guidance provided in Applicants' disclosure. Thus, Murphy et al. teaches at page 22, last paragraph, to page 23, as follows:

> First, live RSV vaccines would be anticipated to stimulate an immune response that resembles the response to wild-type virus infection, including the induction of serum and mucosal antibodies that are able to protect both the upper and lower respiratory tract, as well as the stimulation of a balanced immune response. Second, infection and immunization in the presence of maternal antibodies is possible, since wild-type RSV can infect and replicate efficiently in infants possessing substantial titers of residual maternally-acquired serum antibodies. Third, immunization of RSV-seronegative infants with live attenuated RSV vaccines was not associated with disease potentiation during subsequent natural RSV infection in the vacinees.
>
> How will a live attenuated vaccine induce resistance when wild-type infection itself, in some instances, fails to prevent serious illness during a second infection, especially considering that the immunity induced by live attenuated virus infection is likely to be weaker than that induced by wild-type virus infection? There are two answers to this important question. First, because a single dose of a live virus vaccine will not be sufficient to achieve a high level of immunity, the vaccine will need to be given several times during the first few months of life. Data in the literature suggest two infections with wild-type virus are needed to ensure a durable serum and local antibody response, and this is a partial explanation for the greatly decreased incidence of severe RSV desease during a third or subsequent RSV infection. . . . The optimal schedule will have to be determined by experimentation, but it might require two immunizations within the first two months of life. Fortunately, the immunization procedure will be trivial, involving the application of nose drops containing live vaccine virus. Second, since two antigenically distinct subgroups of RSV exist, the live RSV vaccine will likely be a bivalent vaccine. Reinfection with disease reflects not only waning immunity to the first RSV infection, but also antigenic diversity when the second infection virus is of the heterologous RSV subtype. Thus, multiple administrations of a bivalent RSV subgroup A and B vaccine during the first several years of life will be needed to induce a sustained level of serum and mucosal antibodies that will protect against severe RSV bronchiolitis and pneumonia in infancy and early childhood. (citations omitted, underscores added).

In summary, Murphy et al. do not forecast that development of a live-attenuated RSV vaccine would be attended by "undue experimentation." On the contrary, the solutions offered by Murphy et al. fall directly in line with Applicants' teachings and are squarely within the grasp of the skilled immunologist to implement. For example, the presence of maternal antibodies will be addressed by booster immunization in refractory patient populations. This

course of clinical refinement is clearly disclosed in Applicants' specification. In addition, the specification provides extensive, detailed guidance on how to achieve a fine-tuned balance between attenuation and immunogenicity for the claimed vaccine candidates, and how this balance will be manipulated and validated for different patient populations.

With respect to the existence of multiple, antigenically diverse RSV strains, this variation by no means represents an "undue" challenge in the highly skilled disciplines of virology and vaccine development. On the contrary, the Murphy et al. reference closely accords with Applicant's teachings on this subject, which guide the artisan toward a multivalent vaccine strategy that combines both RSV A and RSV B specific components within a single vaccine formulation or coordinate administration protocol.

The burden is on the Office to establish a *prima facie* case of nonenablement against Applicants' vaccine-related claims, and this burden is substantial. It is not sufficient to cite a handful of obstacles that might preclude a comprehensively effective, or optimal vaccine within the scope of the claims presented for review. Rather, the Office must demonstrate that Applicants' disclosure, complemented by available knowledge and skill in the art, is facially inadequate to enable the artisan to practice the invention in a manner "reasonably commensurate with the scope of the claims" without "undue experimentation."

As emphasized by the Federal Circuit's predecessor court in In re Marzocchi et al. (169 USPQ 367 CCPA 1971), all patent disclosures are entitled to a presumption that they satisfy the enablement requirement. This presumption is only overcome by scientific evidence that is "inconsistent with" the disclosure's teachings. . As further explained in the PTO's Enablement Guidelines, (see, e.g. Example 5E: "Peptides for Treating Obesity"):

> The Office must accept as being true the statements supporting enablement unless there is an objective reason, usually supported with documentary evidence, to question them.

Enablement is also not defeated by a requirement for some experimentation to practice an invention in the manner claimed. In fact, "a considerable amount of experimentation is permissible," so long as the experimentation is not "undue." Ex parte Jackson, 217 USPQ 804, 807 (Bd. Pat. App. Int. 1982). The determination of what constitutes undue experimentation in a

given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. This test is not merely quantitative, and a considerable amount of experimentation is permissible--provided that the specification offers a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. (id., citations omitted). As explained in the case of In re Michalek, 34 CCPA 1124 (1947):

> Skilled workers would as a matter of course, in our opinion, if they do not immediately obtain desired results, make certain experiments and adaptations . . ..

Likewise, in the case of In re Angstadt and Griffin, 190 USPQ 214, 219 (CCPA 1976), the court emphasized that:

> In this art the performance of trial runs using different catalysts is "reasonable," even if the end result is uncertain, and we see no reason on this record why appellants should not be able to claim as their invention the broad range of processes which they have discovered. (emphasis supplied).

With respect to the issue of animal model data, the Office acknowledges that the disclosure provides recombinant RSVs that exhibit desired phenotypic activities of attenuation and immunogenicity sufficient to elicit an immunogenic response in murine subjects. Nonetheless, the Office asserts that the disclosure "does not teach that the immunogenic response is protective in humans against subsequent RSV infection in the presence of passively acquired maternal antibodies or that it is protective against multiple strains of human RSV." Issues raised by this statement have largely been addressed in the preceding paragraphs. The additional question with regard to the predictive value of animal model data is therefore the sole remaining enablement issue that calls for resolution.

To clarify this issue, Applicants note again that the burden to establish nonenablement of the subject claims is on the Office. In this regard, the Office's Enablement Guidelines state, at section III(A)(2), that:

> Since the initial burden is on the examiner to give reasons for the lack of enablement, when possible to supported (sic) by evidence, the examiner must also give reasons for a conclusion of lack of correlation for an in vitro or in vivo animal model example. A

> rigorous or an invariable exact correlation is not required. (citing Cross v. Lisuka, 224 USPQ 739, 747 (Fed. Cir. 1985), emphasis added).

It is clear from this authority that Applicants' murine model data need not be precisely reflective of the activity of a recombinant RSV vaccine candidate in humans in order to fulfill the enablement requirement. On the contrary, the PTO's Enablement Guidelines (see, e.g., Example 5E: "Peptides for Treating Obesity," at page 46) further specify that data from animal testing merely must be "reasonably correlative to treatment in other mammals . . . based on the state of the art."

To further clarify this issue, Applicants respectfully direct the Office's attention to the holding in Ex parte Aggarwal, 23 USPQ2d 1334 (Bd. Pat. Appl. Inter. 1992) where the Board noted:

> Case law subsequent to Brenner is receptive to early filing of applications in the biomedical field so long as the patent applicant, when properly challenged by the examiner, can provide evidence showing substantial activity in screening tests customarily used and accepted as predictive of human activity . . .. All that is required is that there be a "reasonable correlation" between the animal model results and projected activity in humans. (referencing Brenner v. Manson, 383 U.S. 519 (1966), emphasis added).

Applying this authority to the present facts, Applicants have disclosed the use and results of customary assays employing *in vitro* and murine model subjects to demonstrate key phenotypic characteristics of the recombinant RSV vaccine candidates of the invention. These characteristics thus demonstrated in these model systems, namely viability, attenuation, and immunogenicity, each are accepted in the art as correlating reasonably with the corresponding activity of RSV vaccine candidates in humans.

A brief review of the published literature clearly validates the use of murine models to predict general activities of RSV vaccine candidates in humans. RSV model data conventionally follows a progression of in vitro assays, followed by rodent model studies, followed by non-human primate trials, and human clinical trials. Reflective of this widely accepted course of validation, Kim et al. (Pediatrics 52:56/72-62/78, 1973, copy enclosed) report

that a temperature sensitive (*ts*) RSV mutant was selected for human studies based on *in vitro* and subsequent rodent trials, leading to the human testing. In particular, the reference states that:

> This strain, designated as RS-A2, ts-1, was chosen on the basis of its behavior *in vitro* in tissue culture and in vivo in the hamster host and in human volunteers. This mutant did not produce plaques, i.e., did not initiate foci of infection, at or above 37C in cell culture, unlike wild type virus which produced plaques without restriction at 39C. In the hamster, infection with the mutant was limited to the cooler upper respiratory tract (32C to 34C) and virus was not found in the lungs where the temperature was 37C. . . . The mutant infected adult volunteers when administered into the nasopharynx without producing disease and induced resistance to subsequent challenge with virulent wild type virus. (page 56/72-57/73)

From this report, it is clear that *in vitro* and rodent subjects are art-accepted models for general prediction of RSV characteristics in humans, and that data from these models are "reasonably correlative" with activity (e.g., attenuation and immunogenicity) in humans.

A subsequent report by Murphy et al., (Virus Res. 32:13-36, 1994, copy enclosed), details a similar course of validation testing for RSV vaccine candidates (including the biologically derived cpts-530, and cpts-248 strains bearing attenuating point mutations that were identified and incorporated by Applicants into recombinant RSV vaccine candidates)

> Nine mutants of *cp*-RSV, which had acquired either the ts or small plaque (*sp*) phenotype, were generated by chemical mutagenesis with 5-fluoracil. The two ts mutants with the lowest in vitro shut-off temperature, namely the *cpts*-248 (38°C) and *cpts*-530 (39°C) mutants, were the most restricted of the nine *cp*-RSV mutant progeny in replication in Balb/c mice. In seronegative chimpanzees, the *cpts*-248 mutant replicated 4-fold less efficiently in the nasopharynx and cause significantly less rhinorrea than its *cp*-RSV parent. The *cpts*-248 mutant virus, like its sp-RSV parent, was 1000-fold restricted in replication in the trachea compared to wild-type RSV. . . . The *cpts*-248 mutant was immunogenic and induces a high level of resistance in chimpanzees to subsequent challenge with wild-type RSV. The *cpts*-248 mutant therefore exhibits a set of properties that make it a promising vaccine candidate. (page 25, first full paragraph)

In a related report from the same lab, Crowe et al. (Vaccine 13:847-855, 1995,

copy enclosed) present additional studies tracking characteristics of viability, attenuation, and immunogenicity between *in vitro* subjects, BALB/c mice, seronegative chimpanzees, and chimpanzees infused with RSV antibodies prior to immunization. The data from these trials are clearly presented as reasonably correlative between the different model subjects for the subject activities.

The art clearly accepts chimpanzees as closely faithful model subjects to humans with respect to their permissiveness and responses to RSV infections, and this has not been challenged by the Office. The further validation of the tractability between murine, through chimpanzee, to human subjects is nonetheless well documented in the literature. For example, the foregoing validation trials have been carried forward in a related report by Wright et al. (J. Infect. Dis. 182:1331-1342, 2000) detailing clinical trials for a multiply-attenuated RSV vaccine candidate, related to cpts-248, designated cpts-248/404 (also bearing attenuating point mutations that were identified and incorporated into recombinant RSV vaccine candidates by Applicants). In this report:

> A live-attenuated, intranasal respiratory syncytial virus (RSV) candidate vaccine, cpts-248/404 was tested in phase 1 trials in 114 children, including 37 1-2-month-old infants—a target age for RSV vaccines. The cpts-248/404 vaccine was infectious at 10^4 and 10^5 plaque-forming units in RSV-naïve children and was broadly immunogenic in children 6 months old. . . . [t]here was restricted virus shedding on challenge with a second vaccine dose and preliminary evidence for protection from symptomatic disease on natural reexposure. (Abstract).

With respect to the instantly claimed mutants of RSV having modifications of cis-acting regulatory sequences and introductions of stop codons to alter or ablate expression of selected gens, the foregoing evidence relating to *ts*-attenuated RSV also validates the use of animal models for these RSV vaccine candidates, particularly when these candidates are described as being further modified by, e.g., incorporation of *ts* and other desired mutations set forth in the specification.

Applicants' *in vitro* and murine data for the presently claimed recombinant viruses are therefore respectively submitted to be reasonably correlated with the projected

activity of the viruses in non-human primate and human subjects. It is the Office's burden to provide direct evidence contrary to this assertion in order to sustain the instant enablement rejection. The foregoing reports clearly evince that murine model studies are widely accepted in the art as reasonably predictive of RSV activity in non-human primate and human subjects. In this regard, the precise level of viability, attenuation and/or immunogenicity is not at issue. On the contrary, the artisan is mindful of consistent, predictable differences between each accepted animal model subject, and between these models and human subjects. Thus, mice and other rodent model subjects are known to be considerably less permissive for RSV infection than, for example, chimpanzees and humans. This knowledge complements the artisan's ability to extrapolate findings between different subject populations. Thus, while Applicant's animal model data may not be "conclusive" of specific vaccine efficacy for all populations of humans and covering all RSV subtypes and strains, they are clearly "reasonably correlative to treatment in other mammals . . . based on the state of the art." (PTO Enablement Guidelines, supra).

In summary, Applicants submit that the level of skill in the present arts of molecular immunology and vaccine development is high. The skilled artisan is equipped with extensive tools and training with which to implement and adapt Applicants' teachings to produce and select operable vaccine candidates that are fully commensurate with the scope of the claims presented. This guidance provided by Applicants' disclosure contemplates all necessary steps to refine the presently claimed RSV vaccine candidates for use in single or multiple patient populations, including seronegative and seropositive infants, and against single or multiple RSV subgroups, without undue experimentation. For these reasons, withdrawal of the rejection of claims 117-124 under 35 U.S.C. § 112, first paragraph, is earnestly solicited.

Patentability Under 35 USC § 103

Claims 63, 93, 94, 114, 115, 117, 121, 122-124, 127, 131, and 133 are rejected under 35 U.S.C. 103(a) as being unpatentable over Collins et al. (Proc. Natl. Acad. Sci USA, 92:11563-11567, Dec. 1995) in view of any of Marr et al. (Virology, 180/1:400-405, 1991), Chen et al., (Journal of Virology, 67/3:1218-1226, 1993), or Doyle et al. (Journal of Cell Biology, 103/4:1193-1204, 1986).

The Office characterizes the present invention as being drawn to an isolated

infectious recombinant respiratory syncytial virus (RSV) comprising a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase (L) protein, and an RNA polymerase elongation factor, or to an isolated polynucleotide, wherein a modification is introduced within the genome or antigenome comprising introduction of one or more translation termination codons for reduction or ablation of a selected gene. The invention is further characterized to embrace compositions comprising the recombinant RSV virus or polynucleotide and to methods for stimulating the immune system of an individual comprising administering the virus.

The Office cites Collins for allegedly teaching infectious recombinant RSV comprising a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase (L) protein, and an RNA polymerase elongation factor, "wherein defined changes can be introduced for development of live attenuated vaccine strains". Collins is further relied upon for allegedly teaching "that mutations can be introduced that ablate or reduce the level of expression of specific proteins", and for allegedly suggesting "that ablation or modification of specific genes may result in attenuated RSV vaccine strains with enhanced immunogenicity and a higher level of protection against RSV infection than wild-type virus." (citing page 11566, paragraph bridging columns 1 and 2 and page 11567, last paragraph). The Office notes that Collins does not teach modification or ablation of specific genes by introduction of a translation termination codon, as in the present invention. As secondary references, the Office relies upon any of Marr et al., Chen et al., or Doyle et al., as allegedly teaching "mutagenesis of viral genomes by introduction of one or more translation termination codons in order to reduce or ablate expression of specific proteins". Considering these references in view of Collins et al., the Office asserts that it would have been "prima facie obvious to have used the well-known method of mutagenizing a viral genome by introduction of one or more translation termination codons, as is taught by any of Marr et al., Chen et al., or Doyle et al., in order to reduce or ablate expression of one or more genes for generation of attenuated recombinant RSV suitable for use as a vaccine strain."

Applicants respectfully traverse the foregoing grounds for rejection and submit that the subject matter of claims 63, 93, 94, 114, 115, 117, 121, 122-124, 127, 131, and 133 is not rendered obvious by the teachings of Collins et al. in view of Marr et al., Chen et al., or

Doyle et al.

Applicants respectfully submit that the Office's construction of the Collins et al. reference is inconsistent with the actual teachings of the reference as they would be read by the artisan of ordinary skill in the art. In particular, the Office construes Collins et al. as directly teaching "that mutations can be introduced that ablate or reduce the level of expression of specific proteins." This interpretation presupposes that Collins et al. actually forecast a reasonable expectation of success for introducing significant genetic changes (e.g., attenuating point mutations or gene ablations), to yield live-attenuated vaccine candidates. This supposition is directly contravened by the full teachings of the cited reference.

In particular, Collins et al. teach that defined mutations may be introduced into a recombinant RSV. However, the reference prophesies a myriad of possible permutations in recombinant RSV, and does not forecast with any specificity or actual working examples what changes will be effective, nor what the actual result of such changes would be. Thus, the statements in the reference that are relied upon by the Office, at best, suggest that it may be "obvious to try" a broad laundry list of potential mutations in recombinant RSV with the hope of determining a fruitful path for further investigation.

As such, the reference fails to provide the requisite "practical motivation" and specific guidance to raise the disclosure beyond what the courts have characterized as "an invitation to experiment." This interpretation is validated by more detailed teachings within the Collins et al. reference, as set forth in the Discussion section of the article. In particular, the reference states at page 1156 (left column last paragraph, bridging to right column) that:

> The ability to introduce defined mutations into infectious RSV should have many applications in extending analyses of RSV molecular biology and pathogenesis. For example, the functions of the RSV proteins, especially the NS1, NS2, SH, M2(ORF1), and M2(ORF2) proteins, could be investigated by introducing mutations that ablate or reduce their level of expression or that yield mutant protein. (emphasis supplied).

Additional teachings of Collins et al. further clarify that this report was intended, and understood in the art, to provide only an invitation to experiment--leaving it to future investigations to provide specific, practical directions and guidance to arrive at actual working

embodiments. Exemplifying this message, the authors conclude their Discussion with the following statements: "An exciting possibility is that RSV might be engineered in ways that enhance its immunogenicity and induce a level of protection greater than that provided by natural infection." (page 1167, last paragraph) "Also, it should be possible to explore other methods of attenuation." (page 1165, right column, last partial paragraph). (underscore added).

These teachings clearly reflect an "obvious to try" form of disclosure. As articulated by the District Court in Merck and Co. Inc. v. Danbury Pharacal, Inc., 8 USPQ2d 1793, 1816 (D. Del. 1988) (quoting and citing, respectively, In re Fine, 5 USPQ2d 1596, 1599, (Fed. Cir. 1988), and In re Merck, 231 USPQ 375, 379-80 (Fed. Cir. 1986)):

> [T]he governing standard is emphatically not whether a particular methods or process leading to an invention would be "obvious to try", but whether such an experiment would have been expected to succeed.

To determine what constitutes a "reasonable expectation of success" in this context, the Federal Circuit's predecessor court stated in In re Gyurik, 201 USPQ 552, 557 (CCPA 1979) that:

> An element in determining obviousness of a new chemical compound is the motivation of one having ordinary skill in the art to make it. That motivation is not abstract, but practical, and is always related to the properties or uses one skilled in the art would expect the compound to have, if made.

In the instant case, the Office relies upon Collins et al. for allegedly teaching that "mutations can be introduced that ablate or reduce the level of expression of specific proteins." This construction infers a definite disclosure of modifications that could be made in a recombinant RSV, and further supposes that there is a scientifically reasonable expectation that these specific changes will yield "live attenuated vaccine strains" as disclosed by Applicants.

What in fact the present record shows, is that Collins et al. provide a tentative laundry list of "possible" mutations that "could be investigated" or "should be possible to explore". By these statements, the reference facially precludes a determination of any specific guidance and practical motivation to make the selected changes specified in Applicants' claims. Nor does the reference provide positive evidence or specific guidance to convince the artisan to

undertake the manipulations provided as working examples in Applicants' disclosure (e.g., identifying and then constructing and testing deletions of genes having entirely unknown functions)—with the necessary "reasonable expectation" that such significant changes would lead to successful recovery of viable, attenuated, immunogenic vaccine candidates.

Applying controlling legal authority to the foregoing facts, it is clear that the Office's alleged case of *prima facie* obviousness fails. The Collins et al. reference does not teach with practical and specific motivation the particular mutations and combinations of mutations covered by Applicants' claims. Similarly, the reference fails to provide a reasonable expectation of success that such changes would yield the particular results disclosed in Applicants specification. This fact scenario squarely fits the analysis provided by the Federal Circuit in In re O'Farrell,

> [i]n some cases, what would have been "obvious to try" would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication or which parameters were critical or no direction as to which of many possible choices is likely to be successful." 7 USPQ2d 1673, 1681 (Fed. Cir. 1988).

In view of the foregoing evidence and legal authority, Applicants respectfully request that the rejection of claims 63, 93, 94, 114, 115, 117, 121, 122-124, 127, 131, and 133 under 35 U.S.C. 103(a) over Collins et al. in view of Marr et al., Chen et al., and/or Doyle et al. be withdrawn.

Claims 90, 100-101, and 103 are rejected under 35 U.S.C. 103(a) as allegedly unpatentable over Collins et al. in view of any of Marr et al., Chen et al., or Doyle et al. and further in view of any of Crowe et al. (Vaccine 12/8:691-699, 1994, hereinafter Crowe 1994 # 1), Crowe et al. (Vaccine 12/9:783-790, hereinafter Crowe 1994 #2), Crowe et al. (Vaccine 13/9:847-855, hereinafter Crowe et al. 1995) or Murphy et al. (WO 93/21310).

To support this rejection, the Office relies on the primary teachings of Collins et al. as allegedly disclosing "infectious recombinant RSV comprising mutations that ablate or reduce the level of expression of specific proteins as improved vaccine strains." Marr et al., Chen et al., or Doyle et al. are cited for allegedly teaching mutagenesis of viral genomes by

introduction of one or more translation termination codons for reduction or ablation of protein expression. Additional teachings of Collins et al. are asserted pertaining to "biologically derived mutant RSV strains" (see page 11566, column 2, first full paragraph), although the Office notes that Collins et al. does not disclose any of the recited panel of mutant strains. In this context, the Office relies on Crowe 1994 #1 as allegedly teaching cpts RSV 248, on Crowe 1994 #2 for allegedly teaching cpts RSV 248/404, on Crowe 1995 for allegedly teaching cpts RSV 530 and cpts RSV 530/1009, and on Murphy et al. as allegedly teaching cpts RSV 248, 248/404, 248/955, 530, 530/1009, and cpts RSV 530/1030. Further pertaining to these secondary references, the Office asserts that "[e]ach teaches that the biologically derived mutant strains are immunogenic and attenuated." On these grounds the Office contends that it would have been prima facie obvious "to have incorporated attenuating mutations present within one or more of the biologically derived mutant RSV strains taught by Crowe 1994 #1, Crowe 1994 #2, Crowe 1995, or Murphy et al. in order to further attenuate the infectious recombinant RSV virus taught by Collins et al. in view of any of Marr et al., Chen et al., or Doyle et al."

Applicants respectfully traverse this rejection on the basis that Collins et al. is clearly a defective primary reference and does not disclose any "infectious recombinant RSV" as alleged by the Office. The reasons in support of this position are set forth in detail above. For these reasons, the proposed combination of an infectious recombinant RSV vaccine, as allegedly taught by Collins et al. but actually not provided by that disclosure, with the cited secondary teachings does not provide an adequate basis to support the rejection of the subject dependent claims. Withdrawal of the rejection of claims 122, 129, and 130 under 35 U.S.C. 103(a) is therefore earnestly solicited.

Claims 116, 118-120, and 123-124 are rejected under 35 U.S.C. 103(a) as allegedly unpatentable over Collins et al. in view of any of Marr et al., Chen et al., or Doyle et al. and further in view of Randolph et al. (EPA 0 567 100). Collins et al. is relied upon as the primary reference as set forth above. Marr et al., Chen et al., and Doyle et al. are relied upon as secondary references as also set forth above. Randolph et al. is cited for allegedly teachihng intranasal administration of an aerosol containing 106 PFU of attenuated infectious RSV for eliciting systemic immunity (see page 3, lines 1-4; page 6, lines 110; and page 47, Table 19). On this basis, the Office contends that it would have been obvious "to have administered the

recombinant RSV taught by Collins et al. via the dosage and route taught by Randolph because Randolph teaches that the dose and method are effective for eliciting systemic immunity to RSV infection."

Applicants respectfully traverse this rejection on the basis that Collins et al. is a defective primary reference and does not disclose the recombinant RSV as alleged by the Office. The reasons in support of this position are set forth in detail above. For these reasons, the proposed combination of the RSV vaccine as allegedly taught by Collins et al. with a delivery mode as allegedly taught by Randolph et al. is obviated as a basis for rejecting the subject claims. Withdrawal of the rejection of claims 122, 129, and 130 under 35 U.S.C. 103(a) is therefore earnestly solicited.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-467-9600.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned **"Version with markings to show changes made."**

Date: March 25, 2002

Respectfully submitted,

Jeffrey J. King
Registration No. 38,515

WOODCOCK WASHBURN LLP
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Telephone: (215) 568-3100
Facsimile: (215) 568-3439

VERSION WITH MARKINGS TO SHOW CHANGES MADE

63. An isolated infectious recombinant respiratory syncytial virus (RSV) comprising a RSV genome or antigenome, a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase protein (L), and a RNA polymerase elongation factor, wherein a modification is introduced within the genome or antigenome comprising a deletion, insertion, substitution, rearrangement, or nucleotide modification of a cis-acting regulatory sequence within the recombinant RSV genome or antigenome.

64. The recombinant RSV of claim 63, wherein the cis-acting regulatory sequence is a gene-start (GS) signal or a (GE) signal.

65. The recombinant RSV of claim 64, wherein a GS or GE signal is deleted or inserted in the genome or antigenome.

66. The recombinant RSV of claim 64, wherein a GS or GE signal is substituted in the genome or antigenome by a heterologous GS or GE sequence.

67. The recombinant RSV of claim 66, wherein the heterologous GS or GE sequence is of a different RSV gene.

68. The recombinant RSV of claim 67, wherein a GE signal of the RSV NS1 or NS2 gene is replaced by a corresponding GE sequence of the RSV N gene.

69. The recombinant RSV of claim 66, wherein the heterologous GS or GE sequence is of a heterologous negative stranded virus.

70. The recombinant RSV of claim 69, wherein the heterologous GS or GE sequence is of a human RSV A or RSV B subgroup.

71. The recombinant RSV of claim 69, wherein the heterologous GS or GE sequence is of a non-human RSV.

72. The recombinant RSV of claim 70, wherein the heterologous GS or GE sequence is of a bovine RSV.

73. The recombinant RSV of claim 70, wherein the heterologous GS or GE sequence is of a parainfluenza virus (PIV).

74. The recombinant RSV of claim 73, wherein the heterologous GS or GE sequence is of a PIV3 virus.

75. The recombinant RSV of claim 64, wherein a nucleotide sequence of a gene-start (GS) or gene-end (GE) signal is altered in the genome or antigenome.

76. The recombinant RSV of claim 64, wherein a gene-start (GS) or gene-end (GE) signal is rearranged by changing a position of the (GS) or gene-end (GE) signal in the recombinant genome or antigenome.

90. (Amended) The recombinant RSV of claim 63, wherein a [further] modification is introduced within the recombinant genome or antigenome comprising a partial or complete gene deletion, a change in gene position, or one or more nucleotide change(s) that modulate expression of a selected gene.

91. The recombinant RSV of claim 90, wherein a RSV gene is deleted in whole or in part.

92. The recombinant RSV of claim 91, wherein a SH, NS1, NS2, or G gene is deleted in whole or in part.

93. The recombinant RSV of claim 90, wherein expression of a selected RSV gene is reduced or ablated by introduction of one or more translation termination codons.

94. The recombinant RSV of claim 90, wherein expression of a selected RSV gene is reduced or ablated by introduction of multiple translation termination codons.

95. The recombinant RSV of claim 90, wherein expression of a selected RSV gene is reduced or ablated by introduction of a frame shift mutation in the gene.

96. The recombinant RSV of claim 90, wherein expression of a selected RSV gene is modulated by introduction, modification or ablation of a translational start site within the gene.

97. The recombinant RSV of claim 90, wherein a position of one or more gene(s) in the recombinant genome or antigenome is altered relative to a RSV promoter.

98. The recombinant RSV of claim 97, wherein said position of said one or more gene(s) is changed to a more promoter-proximal or promoter-distal location by deletion or insertion of a coding or non-coding polynucleotide sequence within the recombinant genome or antigenome upstream of said one or more gene(s).

99. The recombinant RSV of claim 97, wherein positions of multiple genes in the recombinant genome or antigenome are altered by changing their relative gene order.

100. The recombinant RSV of claim 63, wherein the recombinant genome or antigenome is further modified to incorporate one or more attenuating mutation(s) present in one or more biologically derived mutant human RSV strain(s).

101. (Amended) The recombinant RSV of claim 100, wherein the recombinant genome or antigenome is further modified to incorporate at least one and up to a full complement of attenuating mutations present within a panel of biologically derived mutant human RSV strains, said panel comprising cpts [RSV 248 9ATCC VR 2450)] RSV 248 (ATCC VR 2450), cpts RSV 248/404 (ATCC VR 2454), cpts RSV 248/955 (ATCC VR 2453), cpts RSV 530 (ATCC VR 2452), cpts RSV 530/1009 (ATCC VR 2451), cpts RSV 530/1030 (ATCC VR 2455), RSV B-1 cp52/2B5 (ATCC VR 2542), and RSV B-1 cp-23 (ATCC VR 2579).

102. The recombinant RSV of claim 100, wherein the recombinant genome or antigenome is further modified to incorporate at least one and up to a full complement of attenuating mutations specifying an amino acid substitution at Val267 in the RSV N gene, Glu218 and/or Thr523 in the RSV F gene, Cys319 Phe 521, Gln831, Met1169, Tyr1321 and/or His 1690 in the RSV polymerase gene L, and a nucleotide substitution in the gene-start sequence of gene M2.

103. The recombinant RSV of claim 100, wherein the recombinant genome or antigenome incorporates at least two attenuating mutations.

104. The recombinant RSV of claim 63, wherein the recombinant genome or antigenome comprises a partial or complete human RSV genome or antigenome of one RSV subgroup or strain combined with a heterologous gene or gene segment from a different, human or non-human RSV subgroup or strain to form a chimeric genome or antigenome.

105. The recombinant RSV of claim 104, wherein the heterologous gene or gene segment is from a human RSV subgroup A, human RSV subgroup B, bovine RSV, or murine RSV.

106. The recombinant RSV of claim 104, wherein the chimeric genome or antigenome comprises a partial or complete human RSV A subgroup genome or antigenome combined with a heterologous gene or gene segment encoding a RSV F, G or SH glycoprotein or a cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope thereof from a human RSV B subgroup virus.

107. The chimeric RSV of claim 106, wherein both human RSV B subgroup glycoprotein genes F and G are substituted to replace counterpart F and G glycoprotein genes in a partial RSV A genome or antigenome.

108. The recombinant RSV of claim 106, wherein the chimeric genome or antigenome comprises a partial or complete human RSV B subgroup genome or antigenome combined with a heterologous gene or gene segment from a human RSV A subgroup virus.

109. The recombinant RSV of claim 104, wherein the chimeric genome or antigenome comprises a partial or complete RSV background genome or antigenome of a human or bovine RSV combined with a heterologous gene or genome segment of a different RSV to form a human-bovine chimeric RSV genome or antigenome.

110. The recombinant RSV of claim 63, wherein the recombinant genome or antigenome incorporates a heterologous gene or genome segment from parainfluenza virus (PIV).

111. The recombinant RSV of claim 110, wherein the gene or genome segment encodes a PIV HN or F glycoprotein or immunogenic domain or epitope thereof.

112. The recombinant RSV of claim 110, wherein the genome segment encodes one or more immunogenic protein(s), protein domain(s) or epitope(s) HPIV1, HPIV2, and/or HPIV3.

113. The recombinant RSV of claim 63, wherein the recombinant genome or antigenome is further modified to encode a non-RSV molecule selected from a cytokine, a T-helper epitope, or a protein of a microbial pathogen capable of eliciting a protective immune response in a mammalian host.

114. (Amended) The recombinant RSV of claim 63 which is a complete virus.

115. The recombinant RSV of claim 63 which is a subviral particle.

116. (Amended) The recombinant RSV of claim 63, formulated in a dose of [103 to 106] 10^3 to 10^6 PFU of attenuated virus.

117. A method for stimulating the immune system of an individual to induce protection against respiratory syncytial virus, which comprises administering to the individual an immunologically sufficient amount of the isolated attenuated recombinant RSV of claim 1.

118. The method of claim 117, wherein the recombinant virus is administered in a dose of 103 to 106 PFU of the attenuated RSV.

119. The method of claim 117, wherein the recombinant virus is administered to the upper respiratory tract.

120. The method of claim 119, wherein the recombinant virus is administered by spray, droplet or aerosol.

121. The method of claim 117, wherein the recombinant virus is administered to an individual seronegative for antibodies to RSV or possessing transplacentally acquired maternal antibodies to RSV.

122. A vaccine to induce protection against RSV, which comprises an immunologically sufficient amount of the isolated attenuated recombinant RSV of claim 1 in a physiologically acceptable carrier.

123. The vaccine of claim 122, formulated in a dose of 103 to 106 PFU of the attenuated RSV.

124. The vaccine of claim 122, formulated for administration to the upper respiratory tract by spray, droplet or aerosol.

125. The vaccine of claim 122, wherein the recombinant RSV elicits an immune response against human RSV A, human RSV B, or both.

126. An expression vector comprising an isolated polynucleotide molecule encoding a respiratory syncytial virus (RSV) genome or antigenome modified by a deletion, insertion, substitution, rearrangement, or nucleotide modification of a cis-acting regulatory sequence.

127. An isolated polynucleotide molecule comprising a respiratory syncytial virus (RSV) genome or antigenome which is modified by a deletion, insertion, substitution, rearrangement, or nucleotide modification of a cis-acting regulatory sequence.

128. The isolated polynucleotide molecule of claim 127, wherein the cis-acting regulatory sequence is a gene-start (GS) signal or a (GE) signal.

129. The isolated polynucleotide molecule of claim 127, wherein the cis-acting regulatory sequence occurs within a 3' leader, 5' trailer or intergenic region of the RSV genome or antigenome.

130. The isolated polynucleotide molecule of claim 127, wherein the cis-acting regulatory sequence is a RSV promoter element.

131. The isolated polynucleotide molecule of claim 127, wherein a further modification is introduced within the recombinant genome or antigenome comprising a partial or

complete gene deletion, a change in gene position, or one or more nucleotide change(s) that modulate expression of a selected gene.

132. The isolated polynucleotide molecule of claim 131, wherein a RSV gene is deleted in whole or in part.

133. The isolated polynucleotide molecule of claim 127, wherein expression of a selected RSV gene is reduced or ablated by introduction of one or more translation termination codons in the recombinant genome or antigenome.

134. The isolated polynucleotide molecule of claim 127, wherein expression of a selected RSV gene is reduced or ablated by introduction of a frame shift mutation in the gene.

135. The isolated polynucleotide molecule of claim 127, wherein expression of a selected RSV gene is modulated by introduction, modification or ablation of a translational start site within the gene.

136. The isolated polynucleotide molecule of claim 127, wherein a position of one or more gene(s) in the recombinant genome or antigenome is altered relative to a RSV promoter.

137. The isolated polynucleotide molecule of claim 127, wherein the recombinant genome or antigenome is further modified to incorporate one or more attenuating mutation(s) present in one or more biologically derived mutant human RSV strain(s).

138. The isolated polynucleotide molecule of claim 137, wherein the recombinant genome or antigenome is further modified to incorporate at least one and up to a full complement of attenuating mutations specifying an amino acid substitution at Val267 in the RSV N gene, Glu218 and/or Thr523 in the RSV F gene, Cys319 Phe 521, Gln831, Met1169, Tyr1321 and/or His 1690 in the RSV polymerase gene L, and a nucleotide substitution in the gene-start sequence of gene M2.

139. The isolated polynucleotide molecule of claim 27, wherein the recombinant genome or antigenome comprises a partial or complete human RSV genome or antigenome of one RSV subgroup or strain combined with a heterologous gene or gene segment

American Type Culture Collection

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

NIH
Attn: Peter L. Collins
Bldg. 7, Room 100
7 Center Drive, MSC-0720
Bethesda, MD 10892

Deposited on Behalf of: National Institute of Allergy and Infectious Disease (NIAID)

Identification Reference by Depositor:	**ATCC Designation**
Respiratory Syncytial Virus (RSV) Subgroup B cp23 Clone 1A2	VR-2579

The deposit was accompanied by: ___ a scientific description _ a proposed taxonomic description indicated above.

The deposit was received July 15, 1997 by this International Depository Authority and has been accepted.

AT YOUR REQUEST: X We will not inform you of requests for the strain.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested August 5, 1997. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Date: August 8, 1997

Barbara M. Hailey, Administrator, Patent Depository

cc: Stev n W. Parmal e
Brian Murphy

American Type Culture Collection

12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone: (301)231-5520 Telex: 898-055 ATCCNORTH • FAX: 301-770-1587

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

James E. Crowe, Jr.
National Institutes of Health
Building 7, Room 106
9000 Rockville Pike
Bethesda, MD 20892

Deposited on Behalf of: Laboratory of Infectious Diseases, NIAID, NIH, USPHS and Wyeth-Ayerst Laboratories

Identification Reference by Depositor:	ATCC Designation
Human respiratory syncytial virus mutant:	
RSV A2 cpts-248	VR 2450
RSV A2 cpts-530/1009	VR 2451
RSV A2 cpts-530	VR 2452
RSV A2 cpts-248/955	VR 2453
RSV A2 cpts-248/404	VR 2454

The deposits were accompanied by: __ a scientific description __ a proposed taxonomic description indicated above.

The deposits were received March 22, 1994 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

X We will not inform you of requests for the strains.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested April 18, 1994. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbie A. Brandon Date: June 27, 1994

Bobbie A. Brandon, Head, ATCC Patent Depository

cc: George Tarnowski ✓

BP4

CORRECTED

ATCC

10801 University Blvd • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

James E. Crowe, Jr.
National Institutes of Health
Building 7, Room 106
9000 Rockville Pike
Bethesda, MD 20892

Deposited on Behalf of: Laboratory of Infectious Diseases, NIAID, NIH, USPHS and
Wyeth-Ayerst Laboratories

Identification Reference by Depositor:	**ATCC Designation**
Human respiratory syncytial virus mutant: RSV A2 cpts-530/1030	VR-2455

The deposit was accompanied by: ___ a scientific description _ a proposed taxonomic description indicated above. The deposit was received March 22, 1994 by this International Depository Authority and has been accepted.

AT YOUR REQUEST: X We will not inform you of requests for the strain.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested June 23, 1994. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having auth rity to represent ATCC:

Barbara M. Hailey

Barbara M. Hailey, Administrator, Patent Depository

Date: April 23, 1998

cc: George Tarnowski

American Type Culture Collection

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INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

NIH/NIAID
Attn: Brian R. Murphy
Bldg. 7, Room 106, 7 Center Drive MISC 0720
Bethesda, MD 20892-0720

Deposited on Behalf of: National Institute of Allergy and Infectious Disease

Identification Reference by Depositor: ATCC Designation

Human respiratory syncytial virus (RSV), Subgroup B, Strain B1, cp52 Clone 2B5 VR-2542

The deposit was accompanied by: ___ a scientific description _ a proposed taxonomic description indicated above.

The deposit was received September 26, 1996 by this International Depository Authority and has been accepted.

AT YOUR REQUEST:

X We will not inform you of requests for the strain.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested October 14, 1996. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Barbara M. Hailey

Barbara M. Hailey, Administrator, Patent Depository

Date: October 30, 1996

cc: Steven W. Parmal e

BP4/9

SAFETY AND ANTIGENICITY OF TEMPERATURE SENSITIVE (TS) MUTANT RESPIRATORY SYNCYTIAL VIRUS (RSV) IN INFANTS AND CHILDREN

Hyun Wha Kim, M.D., Julita O. Arrobio, M.D., Carl D. Brandt, Ph.D., Peter Wright, M.D., David Hodes, M.D., Robert M. Chanock, M.D., and Robert H. Parrott, M.D.

Virology Section, Research Foundation of Children's Hospital of the District of Columbia, George Washington University School of Medicine, and the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

ABSTRACT. In attempts to stimulate local respiratory tract immunity to respiratory syncytial virus (RSV), a temperature-sensitive mutant of RSV strain A2 (ts-1 mutant) was administered to 32 infants and children. Evidence of infection was demonstrated in all and no illness accompanied infection in children who had prior experience with RSV. Mild rhinitis occurred in seven infants without prior RSV experience and otitis occurred in one of these individuals. Also, genetically altered virus was recovered from some children but this type of virus represented less than 0.1% of virus recovered from the respiratory tract. An ideal RSV mutant would infect without undergoing such reversion or other genetic change and without producing clinical findings such as otitis which would not be acceptable for a vaccine designed for widespread use.

Pediatrics, 52:56, 1973, VIRUS, RESPIRATORY, VACCINE, RS VIRUS.

SERUM antibody which is of maternal origin or which is stimulated by injection of a highly antigenic, inactivated, respiratory syncytial virus (RSV) vaccine does not protect against RSV disease.[1-3] However, naturally acquired RSV infection confers a definite degree of protection against subsequent RSV illness.[3,4] These findings suggested to us several years ago that local defense mechanisms in the respiratory tract play a major role in resistance to illness caused by RSV.[3] We have, therefore, sought an attenuated strain of RSV which would induce resistance without producing significant illness. In earlier studies we evaluated a low temperature adapted strain which produced a silent infection in infants and children who had been infected with RSV previously.[5] However, mild lower-respiratory tract disease developed in one young infant without prior RSV experience when he was given this potential vaccine virus. Our findings indicated that a measurable degree of attenuation had been achieved but the residual virulence of the low temperature adapted strain made it unacceptable for use in young infants, the group for whom an RS virus vaccine is most urgently needed.

Encouraged by partial success with the low temperature RSV strain, we were anxious to evaluate other candidate vaccine strains which offered the possibility of greater attenuation. One such strain was a temperature-sensitive (ts) mutant induced by the chemical mutagen 5-fluorouridine.[6] This strain, designated as RS-A2, ts-1, was chosen on the basis of its behavior *in vitro* in tissue culture and *in vivo* in the hamster host and in adult volunteers.[7] This mutant did not produce plaques, i.e., did not initiate foci of infection, at or above 37C in cell culture, unlike wild type virus which produced plaques without restriction at 39C. In the hamster, infection with the mutant was limited to the cooler upper respiratory tract (32C to 34C) and virus was not found in the lungs where the temperature

(Received November 2, 1972; revision accepted for publication January 23, 1973.)

These studies supported in part by National Institute of Allergy and Infectious Diseases grant AI01528-13, contract NIH-NIAID-71-2091, and General Clinical Research Center grant 5 M01 FR00284-04.

ADDRESS FOR REPRINTS: (R.H.P.) Children's Hospital of the District of Columbia, 2125 Thirteenth Street, N.W., Washington, D.C. 20009.

TABLE I

EVIDENCE OF INFECTION IN INFANTS AND CHILDREN WHO RECEIVED THE ts-1 MUTANT RESPIRATORY SYNCYTIAL VIRUS (RSV)

Previous RSV Infection*	No. in Group	No. With Indicated Evidence of Infection			
		Virus Recovery	Rise in Neutralizing Antibody		Virus and/or an Immunological Response
			Serum	Nasal Secretion	
No†	7	7	7	7	7
Yes	25	20	7	19	25
Total	32	27	14	26	32

* Based upon presence or absence of detectable plaque reduction antibody in serum (minimum serum dilution, 1:20).

† Includes one infant born after the last RSV epidemic who had a serum titer of 1:64.

was 37C. The temperature-sensitive characteristic of this mutant was stable following growth in cell culture and in hamsters. Thus, there was no evidence of reversion to the wild phenotype. The mutant infected adult volunteers when administered into the nasopharynx without producing disease and induced resistance to subsequent challenge with virulent wild type virus.[5] Finally, the mutant appeared to be genetically stable in man in that isolates obtained from the adult volunteers retained their ts property.

This report will describe the response to the ts-1 mutant of 32 infants and children who were between 6 months and 6 years of age.

METHODS

Two infants or children at a time were admitted to the Clinical Research Center (CRC) and were observed closely for three weeks. They were kept in isolation together in a hospital room and special precautions were taken to prevent their acquiring extraneous infection. In each instance written informed consent was obtained from the parents or guardians after the aims and

TABLE II

RELATIONSHIP OF AGE TO RSV SHEDDING IN RECIPIENTS OF THE ts-1 MUTANT

Age in Years	No. Tested	Pattern of Virus Shedding			
		No. Positive	Time to Initial Shedding (Mean Days)	Duration in Days (Mean)	Maximum Virus Shed*
0.5–1	9	8	5.4	8.6	$10^{3.8}$
1–2	9	9	6.2	4.6	$10^{4.7}$
2–4	7	7	5.3	3.6	$10^{3.8}$†
4–6	7	3	10.0	2.0	NT
Total	32	27	6.1	5.2	$10^{3.8}$

* Throat swab fluid, plaque forming units/ml (geometric mean).

† Only two subjects tested in this group.

TABLE III

RELATIONSHIP OF PREEXISTING SERUM ANTIBODY TO RSV SHEDDING

Serum Neutralizing Antibody Titer Prior to Administration of ts-1 Mutant	No. Tested	Pattern of Virus Shedding			
		No. Positive	Time to Initial Shedding (Mean Days)	Duration in Days (Mean)	Maximum Virus Shed*
<1:20	7†	7†	3.4	10.0	$10^{3.7}$
1:60–1:300	9	8	5.5	5.4	$10^{3.0}$
1:301–1:2100	16	12	8.2	2.3	$10^{1.6}$‡
Total	32	27	6.1	5.2	$10^{3.2}$

* Throat swab fluid, plaque forming units/ml (geometric mean).
† Includes one infant born after the last RS virus epidemic but serum titer of 1:64.
‡ Only seven were tested.

procedure of the study had been explained to them.

Approximately 10^4 $TCID_{50}$ of the ts-1 mutant of RSV was administered into the nose by pipette and into the pharynx by coarse spray. The infants and children were examined at least twice daily and throat swab, anal swab, nasal secretion, and serum samples were obtained and tested as described previously.[3] Serum and nasal secretion neutralizing antibody was measured by the plaque reduction technique. For the last 20 vaccinees in the study the quantity of virus present in throat swab fluid was determined daily for the first 14 days by inoculating freshly collected specimens onto HeLa cell monolayer cultures and counting RSV plaques after a suitable incubation interval. In an attempt to determine whether the ts mutant might have reverted toward wild type virus the HeLa cell plaque assay cultures (inoculated with fresh throat swab fluids) were incubated at 32C (a "permissive" temperature, one which allows growth of the mutant) or 39C (a "restrictive" temperature, one which restricts growth of the mutant). Also, the efficiency of plaque formation of the tissue culture-grown progeny of virus recovered from the vaccinees was determined at 32C (a permissive temperature) and at 37C, 38C, and 39C (restrictive temperatures). In tests for temperature sensitivity HEp-2 or HeLa cell monolayer cultures wer used and the inoculated cultures were incubated in a large water bath in order to assure constancy of temperature control ($\pm$ 0.05 C).

The ts-1 mutant virus was administered to 32 infants and children 6 months to 6 years of age: seven children were 4 to 6 years old, seven children 2 to 4 years old, nine children 1 to 2 years old, and nine infants 6 to 11 months old. It appeared that seven of the vaccinees had not experienced prior RSV infection as indicated by the absence of serum plaque reduction antibody (titer of < 1:20 in six vaccinees) or a low titer considered to be of maternal origin (one vaccinee).

No attempt was made to study the question of spread of infection from vaccinees to cohorts of the same age. However, the nursing staff of the CRC was tested for presence of RSV in their pharynges each week by inoculating throat swab fluid into HEp-2 cell roller tube cultures. These cultures were incubated at a permissive temperature of 33C. In this manner it was hoped that transmission of the ts-1 mutant of RSV to adult contacts could be detected if it occurred.

RESULTS

Evidence of Infection

The ts-1 mutant virus produced infection in 27 of the infants and children as evidenced by recovery of virus from throat swab specimens (Table I). All of the sub-

TABLE IV

SERUM NEUTRALIZING ANTIBODY RESPONSE TO ts-1 MUTANT RSV

Previous RSV Infection	No. in Group	Virus Shed		Time (weeks)	Mean Serum Antibody Titer* (Reciprocal)	Fold Antibody Rise
		Yes or No	Number			
No	7	Yes	7	0	<20	
				2	117	5.8×
				3	407	27×
				7	683	46×
Yes	25	Yes	20	0	350	
				2	711	2×
				3	841	2.4×
				7	736	2.1×
		No	5	0	518	
				2	525	
				3	477	
				7	537	

* Determined by plaque reduction technique.

jects without evidence of prior RSV infection excreted virus. Additional evidence of infection by the vaccine strain was the development of significant increases in serum antibody by 14 of the 32 vaccinees. Interestingly none of the children over 4 years of age showed a serum neutralizing antibody response. Significant (threefold or greater) increases in nasal antibody titer were seen in 26 infants and children.

If virus recovery and/or a significant rise in nasal secretion neutralizing activity is considered evidence of infection, then the ts-1 mutant virus induced infection in 100% of the recipients.

Pattern and Intensity of Infection

Initial excretion of virus was detected as early as the second day and as late as the 20th day after administration of the mutant; the mean time to initial excretion was 6.1 days (Table II). The mean duration of excretion was 5.2 days.

The youngest subjects shed virus earlier and for longer periods and tended to shed virus in greater amounts (Table II). This correlation almost surely reflects the relationship between age and prior experience with the RS virus. Similarly (Table III) those individuals with lower serum neutralizing antibody levels prior to vaccine virus infection shed virus earlier, for a longer period, and in greater amounts than those with higher antibody levels.

The serum antibody response to the ts-1 mutant virus in infants without prior RSV experience was quite marked (Table IV). A geometric mean antibody titer increase of 46-fold occurred as contrasted with a rise of

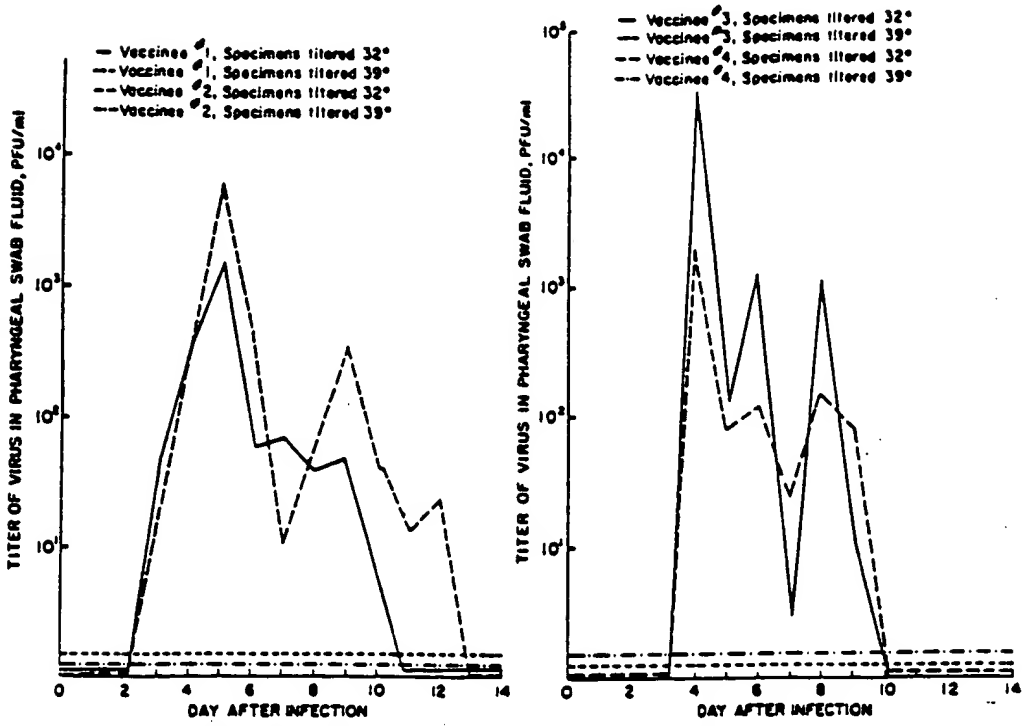
TABLE V

EFFICIENCY OF PLAQUE FORMATION AT PERMISSIVE AND RESTRICTIVE TEMPERATURES OF RS VIRUSES RECOVERED FROM INFANTS AND YOUNG CHILDREN INFECTED WITH ts-1 MUTANT VIRUS—REPRESENTATIVE FINDINGS FROM A SINGLE TEST

RS Virus	Titer per ml in Plaque-Forming Units at Indicated Temperature			
	35°C	37°C	38°C	39°C
Wild type	$10^{6.8}$	$10^{6.6}$	$10^{6.2}$	$10^{6.0}$
ts-1 vaccine*	$10^{6.6}$	$<10^{1.7}$	$<10^{1.7}$	$<10^{1.7}$
Isolate 9151†	$10^{6.6}$	$10^{6.6}$	$10^{6.2}$	$10^{6.0}$
Isolate 8519†	$10^{6.7}$	$10^{6.6}$	$10^{6.4}$	$10^{6.4}$
Isolate 8969†	$10^{6.4}$	$10^{6.4}$	$<10^{1.7}$	$<10^{1.7}$
Isolate 9177†	$10^{6.4}$	$10^{6.4}$	$<10^{1.7}$	$<10^{1.7}$

* Suspension of ts-1 mutant which was administered to infants and young children.

† These isolates were tested as second passage suspensions in HEp-2 cells.



FIGS. 1 and 2. Representative patterns of virus excretion exhibited by four vaccinees who received 10^4 $TCID_{50}$ of the ts-1 mutant and who lacked serological evidence of prior infection with RS virus. Phenotype of the excreted virus was determined by assay for plaque formation at 32C (a permissive temperature) and at 39C (a restrictive temperature).

twofold or less which was seen for those individuals who had prior experience with RSV. These findings suggest that the attenuated RS virus was capable of inducing a significant immune response in previously unconditioned infants.

Reversion

Virus with wild type temperature sensitivity, i.e., capacity to produce plaques in tissue cultures at 37C or higher, was recovered from the tissue culture-grown progeny of virus recovered in HEp-2 cells from pharyngeal swabs of some of the infants and children infected with the ts-1 mutant. Many of these "revertants" were not identical with wild type virus (which exhibits a high efficiency of plaque formation at 37C, 38C, and 39C) in that plaque formation was restricted at 38C or 39C. This suggests that "suppressor mutation" rather than a complete back-mutation to the original wild type had occurred. The temperature sensitivity patterns of some typical isolates with "suppressor mutations" as well as an isolate which appeared to have reverted to wild type are presented in Table V. Each of the isolates was titered at 32C and at the three restrictive temperatures; the wild type virus and the ts-1 vaccine virus were also tested for comparison.

Of a total of 139 isolates recovered in this study, 34 showed evidence of genetic alteration from the ts phenotype. Eight isolates appeared to have completely lost temperature sensitivity (reversion to wild type) whereas 26 exhibited the pattern of temperature sensitivity which we interpret as the result of suppressor mutation.

In toto "revertant" virus or virus with "suppressor mutation" was isolated at some time from 16 of the 27 children who shed

virus. Ten children shed virus with "suppressor mutation," two shed "revertant" virus, and four shed both types of virus. There was no correlation of the shedding of either type of virus with the child's age or serum neutralizing titer. There was, however, a definite tendency for these viruses to appear late in infection. One "suppressor mutatant" appeared on day 3 but none of the other phenotypically altered viruses was shed earlier than day 7, with the average day of appearance being day 11. It was not uncommon to find a "suppressor mutant" or "revertant" virus being shed one day and a temperature-sensitive virus reappearing the next.

It should be emphasized that each of the genetically altered isolates was detected by testing the progeny of virus which had been passaged several times in tissue culture. Under these conditions a small quantity of genetically altered virus could overgrow the more defective ts-1 mutant and thus examination of the tissue culture-grown progeny of an isolate might not yield a reliable estimate of proportion of genetically altered virus shed by a vaccinee. In an attempt to obtain a reliable estimate of reversion frequency the oropharyngeal specimens from 20 vaccinees were titrated directly, without prior freezing and thawing, on HeLa cell monolayers at 32C (a permissive temperature) and 39C (a restrictive temperature). In this manner the proportion of revertant virus shed by vaccinees could be estimated directly.

Revertant virus was detected on only one occasion from one vaccinee and on the next day only ts virus was recovered from this individual. Representative findings of the direct titrations are shown in Figures 1 and 2; these data are derived from four vaccinees who lacked prior experience with RSV. At the peak of virus replication, i.e., the fourth day after infection, revertants represented less than 0.01% to 0.1% of the virus recovered from the respiratory tract. On the fourth day after infection 10^3 to $10^{4.5}$ plaque forming units of virus per ml of oropharyngeal swab fluid were detected at 32C, whereas no plaque was observed at 39C.

These findings suggest that revertant virus did not "take over" and become the dominant species of the viral flora present in the vaccinees. Finally, the occurrence of this low level of "reversion" was not associated with any signs or symptoms of illness.

TABLE VI

CLINICAL RESPONSE TO INFECTION WITH ts-1 MUTANT RSV

Previous RSV Infection	No. in Group	No. Infected	No. With Indicated Response		
			Rhinorrhea	Fever	Otitis Media
No	7	7	7	1	1
Yes	23	23	6	0	0

Communicability

Weekly tests for RSV in the oropharyngeal specimens collected from the nursing staff of the CRC yielded only one RSV isolate. This isolate failed to produce plaques at 39C suggesting that the virus which had spread from a vaccinee to a nurse had retained its ts phenotype. We have tested an isolate kindly supplied by Dr. Kenneth McIntosh which was derived from a child who was a contact of a ts-1 RSV vaccinee and this isolate was also temperature sensitive. This limited experience suggests that the vaccine virus was communicable; however, the virus which infected contacts appeared to be of the temperature-sensitive variety. The latter finding was not surprising since the vast majority of virus shed by vaccinees at the time of peak virus replication was temperature sensitive.

Clinical Effects: Acceptability

No untoward effects or reactions occurred within 48 hours of virus administration. No significant nasopharyngeal, respiratory tract, or febrile illness was detected at the time of virus excretion in any of the individuals with evidence of previous RSV infection, although very mild rhinorrhea was seen in six of these vaccinees (Table VI).

A definite but mild, nonfebrile rhinitis oc-

curred in the seven infants who had not been infected with RS virus prior to receiving the ts-1 mutant. The rhinitis started on the third or fourth day of virus excretion; there was rhin rrhea with mild erythema and edema of nasal mucous membranes. During the period of virus excretion significant fever did not occur. The rhinorrhea lasted for up to 13 days but did not affect the appetite or general well-being of the infants.

In one of these infants significant fever occurred two days after excretion of virus had ceased and this coincided with development of moderate bilateral otitis media. *Diplococcus pneumoniae*, type 6, was recovered from nasal secretions at this time and defervescence occurred within four hours after penicillin G was given intramuscularly. The otitis media also improved rapidly. We are concerned that this otitis media may have resulted from local lymphoid hyperplasia in the nasopharynx and blockage of the eustachian tube, possibly a sequela of the ts-1 RSV infection.

DISCUSSION

In brief, the ts-1 mutant was acceptably attenuated for adults and for children who had prior experience with RSV. In infants with no prior experience, mild rhinitis was induced by the mutant and this would probably be an acceptable price to pay for protection against serious RSV disease of the lower respiratory tract. However, the additional development of otitis media following this rhinitis in one infant is a source of concern which prompts us to consider using a smaller dose of the virus in order to determine whether infection can be induced without initiating significant nasal mucous membrane response.

We would also consider initiating clinical evaluation of other ts mutants of RSV in hopes that a mutant can be identified which will (1) infect without undergoing reversion or other genetic change, (2) induce resistance to wild type virus, and (3) cause no rhinitis or a milder rhinitis than that caused by the ts-1 mutant given at the present dosage level to seronegative infants.

Possibly our concern over genetic alteration of the ts-1 mutant during infection of infants and young children may prove to be unwarranted. Clearly, the small amount of revertant virus produced at the height of the vaccine infection did not pose a threat to the health of our vaccinees. Limited information from two contact infections, involving a nurse in our study and a child studied by Dr. Kenneth McIntosh, indicates that infection with the ts-1 mutant can be communicable. However, the virus which was transmitted was temperature-sensitive in both instances. If future contact infections conform to this pattern, communicability may not constitute a barrier to the use of the ts-1 mutant of RSV.

Although the ts-1 mutant may not be the definitive RSV vaccine strain, this study has been helpful in providing a frame of reference for evaluation of other ts mutants of RSV. We have defined more sharply the properties required for an acceptable vaccine strain and one more attenuated RSV strain has been shown to produce infection and antibody response. Pending the availability of additional RSV strains for evaluation in infants and young children we believe that additional studies in young children with the ts-1 mutant are warranted to determine whether infection can be induced without producing illness following administration of a smaller amount of the virus.

SUMMARY

The consensus is that the most promising approach to immune prophylaxis against respiratory syncytial virus (RSV) disease in infants is to stimulate local respiratory tract antibody with inactivated or attenuated vaccines prior to natural infection. A 26C adapted RSV vaccine was found to have sufficient residual pathogenicity for young infants that we have initiated safety and antigenicity studies with a different candidate attenuated vaccine, a temperature sensitive-mutant of RSV strain A2 (ts-1 mutant) propagated at 30C in primary bovine

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An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines

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Abstract

RSV and PIV3 are responsible for about 30% of severe viral respiratory tract disease leading to hospitilization of infants and children. For this reason, there is a need to develop vaccines effective against these viruses. Since these viruses cause severe disease in early infancy, vaccines must be effective in the presence of maternal antibody. Currently, several strategies for immunization against these viruses are being explored including peptide vaccines, subunit vaccines, vectored vaccines (e.g., vaccinia-RSV or adenovirus-RSV recombinants), and live attenuated virus vaccines. The current status of these approaches is reviewed. In addition, the immunologic basis for the disease potentiation seen in vaccinees immunized with formalin-inactivated RSV during subsequent RSV infection is reviewed. The efficacy of immunization in the presence of maternal antibody is discussed. Much progress for a RSV and PIV3 vaccine has been made and successful immunization against each of these pathogens should be achieved within this decade.

1. Introduction

Much progress has been made in the evaluation of vaccines for RSV and PIV3 since this subject was last reviewed (Murphy, 1988; Murphy et al., 1988b). The purpose of this review is to provide an update on the evaluation of candidate RSV

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and PIV3 vaccines in animals and humans. These two respiratory viruses have been selected for special consideration because they account for approximately 30% of all hospital admissions for pediatric respiratory tract disease, and, therefore, are the prime targets for immunoprophylaxis (Murphy et al., 1988b).

2. Vaccine development for RSV

General considerations

RSV, a paramyxovirus, is the leading agent of severe lower respiratory tract disease in the pediatric population. RSV causes pneumonia and bronchiolitis and is unusual in its ability to infect very young infants, despite the presence of maternally-acquired serum antibodies. Several obstacles have hindered the development of an RSV vaccine. First, because RSV causes severe disease by 2 months of age, immunization should be initiated within the first month of life so that protective levels of immunity are achieved before two months of age (McIntosh and Chanock, 1990). Since most infants in this age group possess maternally acquired anti-RSV serum antibodies, the results obtained from evaluation of experimental vaccines in older seronegative subjects cannot be directly extrapolated to the very young infant. Passively-acquired maternal antibodies to RSV present in the young infant can decrease the immunogenicity of vaccines administered (Murphy et al., 1988a). The immunogenicity of subunit and vectored RSV vaccines administered parenterally is suppressed by maternally-acquired antibodies through a poorly defined immunologic mechanism termed antibody-mediated immune suppression (Murphy et al., 1988a; Murphy et al., 1991). Live attenuated viral vaccines are also affected by passively-acquired antibodies which can decrease the infectivity and, as a consequence, the immunogenicity of the vaccine. This is the situation for the live attenuated measles virus vaccine administered parenterally; the vaccine virus is sensitive to neutralization by the passively-acquired antibodies, and its administration must be delayed until the level of maternal antibody has decreased to an unprotective level. The loss of infectivity of a live virus vaccine mediated by passive serum antibodies can be circumvented by administration of the vaccine by a mucosal route (Murphy et al., 1989a), as has been accomplished successfully for polioviruses. Passively-acquired serum antibodies have limited access to the respiratory or gastrointestinal mucosa and, therefore, infection and immunization can proceed despite their presence in the serum.

The second obstacle to immunization against RSV is that immunity induced by infection with wild-type RSV itself is incomplete since multiple infections are a hallmark of this virus and severe disease can occur even during the second infection (McIntosh and Chanock, 1990). Protection against disease in the lower respiratory tract caused by RSV can be achieved, but two or more infections are often required to accomplish this. Therefore, immunization against RSV, like that against poliovirus, will likely require multiple doses of vaccine to achieve the level of immunity needed to prevent serious disease of the lower respiratory tract. The immunologic mechanisms underlying the partial immunity induced by infection

with wild-type virus are detailed elsewhere (Murphy and Chanock, 1991), but include at least one or more of the following factors: immaturity of the neonatal immune system, antibody-mediated immune suppression, and a relatively rapid decline in the levels of protective mucosal and serum antibodies observed following first infection.

The third obstacle to RSV vaccine development is that two antigenically divergent RSV subgroups exist, namely subgroups A and B, which are about 25% related by cross-neutralization analysis (McIntosh and Chanock, 1990; Johnson et al., 1987; Hendry et al., 1988). Second infections by RSV are often caused by virus belonging to the heterologous subgroup indicating that antigenic diversity is responsible in part for the high frequency of second infections, and more importantly, for lower respiratory disease upon second infection (McIntosh and Chanock, 1990; Muelenaer et al., 1991). An RSV vaccine should protect against both subgroups.

Fourth, during vaccine trials in the 1960's immunization of infants and young children with an inactivated RSV vaccine unexpectedly potentiated RSV disease during subsequent natural infection (discussed in more detail later in this review) (Kapikian et al., 1969; Kim et al., 1969). This observation, in the context of comparable observations for inactivated measles virus vaccine, dictated that vaccine development for RSV proceed with great caution. In summary, an RSV vaccine that successfully overcomes these obstacles will need to: (1) be effective in the presence of maternally-acquired serum antibodies; (2) induce a level of resistance comparable to that of wild-type virus; (3) induce resistance to both subgroup A and B strains of RSV; and (4) not potentiate RSV disease during subsequent natural infection.

RSV subunit vaccine

The success with the development of a hepatitis B surface antigen vaccine using modern molecular virologic techniques generated optimism that such approaches could be rapidly applied to other pathogens, including RSV and PIV3. The major protective antigens of RSV, the fusion (F) and attachment (G) glycoproteins which induce neutralizing and protective antibodies (McIntosh and Chanock, 1990; Connors et al., 1991), are the target antigens for inclusion in an RSV subunit vaccine (Levine et al., 1989; Routledge et al., 1988; Walsh et al., 1987; Wathen et al., 1989b). Initial studies, which were very encouraging, found that rodents could be successfully immunized with isolated F or G glycoprotein antigens and that disease potentiation, as indicated by enhanced pulmonary histopathology, was not observed in immunized animals following RSV challenge (Routledge et al., 1988; Walsh et al., 1987; Murphy et al., 1989b). The immunogenic and protective F or G glycoproteins used in these studies were either purified by physical techniques from virions or by immunoaffinity chromatography from insect cells infected with a baculovirus-F recombinant virus or from RSV-infected mammalian cell lysates (Levine et al., 1989; Routledge et al., 1988; Walsh et al., 1987; Wathen et al., 1989b). An RSV G glycoprotein produced in Salmonella has recently been found to induce neutralizing antibodies in cotton rats (Martin-Gallardo et al., 1993).

Immunization of rodents with F glycoprotein from a subgroup A virus protected against subgroup A or B RSV challenge (Wathen et al., 1989b; Walsh, 1991). Three different forms of RSV F glycoprotein, namely, non-reduced oligomeric F, reduced or monomeric F, or a truncated F lacking the C-terminal anchor sequences, were protective in rodents (Walsh et al., 1987; Wathen et al., 1989b). However, the protection observed was incomplete in that significant resistance to virus replication was seen only in the lower respiratory tract, but the challenge virus replicated efficiently in the upper respiratory tract (Walsh et al., 1987; Murphy et al., 1989b). This pattern of resistance differed from that induced by prior infection with RSV which provided complete protection of both sites of the respiratory tract. These findings support the conclusion that immunization against RSV is most effective when it involves stimulation of local mucosal immunity.

These relatively encouraging findings were soon tempered by the realities of RSV vaccine development. Recent retrospective analysis of sera from the FI–RSV trials in the 1960's showed that immunization-induced antibodies against the F glycoprotein that had high titers of ELISA binding antibodies but low neutralizing activity (Murphy et al., 1986b). This unusual antibody response was subsequently observed in rodents immunized with FI–RSV or with purified F glycoprotein (Murphy et al., 1989b; Murphy et al., 1990). In contrast, the neutralizing activity of RSV F-specific antibodies induced by infection with a vaccinia-RSV recombinant virus expressing the RSV F glycoprotein was quite similar to that induced by wild-type RSV infection (Connors et al., 1992a). These observations strongly suggested that the purification of the F glycoprotein resulted in its altered immunogenicity, presumably by disrupting the conformationally-dependent neutralizing epitopes present in the protein.

An important advance in RSV vaccine development resulted from the development of an experimental model for RSV disease potentiation in which cotton rats immunized with FI–RSV vaccine developed enhanced pulmonary histopathology during subsequent RSV challenge (Prince et al., 1986). This observation in cotton rats is thought to recapitulate, in an experimental animal, the disease potentiation that was observed in the FI–RSV vaccinees in the 1960's. It has since become apparent that the extent of RSV replication in cotton rats is much less than that in humans, and, consequently, the magnitude of immunopathological reactions would be expected to be more limited in scope. Despite this limitation, it now became possible to test new vaccines, such as the F subunit vaccine, against FI–RSV for their ability to cause disease potentiation. Evaluated in this way, immunization with purified F glycoprotein produced in mammalian or insect cells was shown to result in enhanced pulmonary histopathology in cotton rats challenged with RSV 3–6 months following immunization. The pattern and magnitude of the cellular infiltration in the lungs of these RSV-challenged animals was similar to that present in FI–RSV immunized, challenged cotton rats (Murphy et al., 1990; Connors et al., 1992a). In addition, FI–RSV and purified F subunit vaccines (as well as purified G or N protein) can induce enhancement of pulmonary histopathology in mice (Vaux-Peretz et al., 1992). It is important to indicate that the interpretation of findings in cotton rats is controversial (see Hildreth et al.,

1993a, 1993b; Murphy, 1993). Nevertheless, these findings demonstrated that FI–RSV and subunit F preparations could induce a similar pattern of immunological response in animals that resulted in enhanced pulmonary infiltration with inflammatory cells upon challenge with RSV.

An additional concern about purified subunit vaccines is their poor immunogenicity in seronegative human subjects. Also, protein antigens of human pathogens that are highly immunogenic in mice can have greatly reduced immunogenicity in chimpanzees or humans. The high immunogenicity of a vaccinia-F recombinant virus in rodents was not seen in chimpanzees (Collins et al., 1990b). It is therefore possible that subunit vaccines will be weakly immunogenic in seronegative chimpanzees or humans. Earlier studies in rodents showed that the immunogenicity and protective efficacy of purified F glycoprotein could be significantly suppressed by passive transfer of RSV hyperimmune serum prior to immunization (Murphy et al., 1991). This suggested that the poor immunogenicity of the subunit vaccine in immunologically naive young infants would be further diminished by maternally-acquired antibodies. The immunogenicity of the F subunit vaccine in humans was studied by immunization of 35 seropositive young children (12–36 months of age) with 20 μg of F subunit vaccine. It was found to induce F-specific antibodies of low neutralizing activity that failed to prevent illness upon infection with RSV (Tristram et al., 1992). A higher dose of vaccine (50 μg) was immunogenic in seropositive children and possible efficacy against upper respiratory tract illness caused by natural infection with RSV was observed (Tristram et al., 1993). In an additional study in seropositive children, mild upper respiratory tract illness was seen during natural RSV infection in vaccinees who received either 5, 20 or 50 μg of subunit vaccine (Belshe et al., 1993).

Studies with a novel RSV chimeric FG subunit vaccine were conducted in parallel with the F subunit vaccine described above. This vaccine consisted of a purified FG chimeric protein in which the anchor regions of the F (COOH-terminal) and G (NH_2-terminal) glycoproteins were deleted and the two ectodomains of the proteins were expressed as a fused protein with F sequences being at the NH_2-terminal end of the chimera (Wathen et al., 1989a). This protein was expressed by a baculovirus-FG recombinant virus in insect cells and the FG protein, which was secreted into the medium, was purified by immunoaffinity chromatography. The FG subunit vaccine had several properties in common with purified F subunit vaccine. First, immunization with FG glycoprotein was protective in rodents, with protection largely being confined to the lungs (Connors et al., 1992a; Brideau et al., 1989; Wathen et al., 1991). Second, antibodies of low neutralizing activity were induced (Connors et al., 1992a). Third, enhanced pulmonary histopathology was observed in FG-immunized cotton rats following RSV challenge (Connors et al., 1992a). However, immunization of African green monkeys with FG glycoprotein did not induce enhanced pulmonary histopathology upon challenge, but the vaccine was weakly immunogenic and only marginally protective in the lung (Kakuk et al., 1993). In addition, it was found that the immunogenicity of the G component of the FG vaccine was very low (Connors et al., 1992a).

The studies of the immunogenicity of the FG subunit vaccine expanded previous studies of r subunit vaccine in two important ways. First, studies of cell-mediated immunity induced by FG demonstrated that $CD4^+$ T-cells were efficiently induced by immunization with FG, but $CD8^+$ cytotoxic T-cells were not (Brideau et al., 1990; Brideau and Wathen, 1991; Nicholas et al., 1990). This is a similar pattern to that observed for FI-RSV immunization of humans or rodents (Nicholas et al., 1990; Kim et al., 1976; Connors et al., 1992b). Second, the protective activity of passively transferred antibodies induced by immunization with FG that had high-binding/low-neutralizing activity was compared to that of antibodies induced by infection with RSV or by infection with both vaccinia-F and G recombinant viruses (Connors et al., 1992a). Passive transfer of immune sera from animals infected with RSV or with vaccinia-F and vaccinia-G was highly protective, whereas immune sera from animals immunized with FG failed to protect the lungs against RSV challenge (Connors et al., 1992a). These observations demonstrated that antibodies induced by FG that had low-neutralizing activity in vitro also had low functional activity in vivo, i.e., they failed to provide passive protection. Increased pulmonary histopathology was not observed in the passively immunized animals following RSV challenge, suggesting that antibodies were not the major mediator of the inflammatory response. Rather, the histopathology observed in the FG-immunized, RSV-challenged animals appears to be mediated by T-cells (discussed in greater detail below).

Three major characteristics of current formulations of RSV subunit vaccines make them unlikely to be successful vaccines in humans. First, if these vaccines prove to be poorly immunogenic in seronegative chimpanzees and humans, it might not be possible to deliver a sufficient number of doses in the neonatal period [when F antigens are least immunogenic in humans (Walsh, 1991; Murphy et al., 1986a)] to induce a protective level of immunity. Second, antibodies induced by vaccine have a low level of functional activity in vivo and will probably not protect the vaccinee. Third, maternally-acquired serum antibodies can be expected to significantly suppress the immune response to the parenterally-administered vaccine. Given these three factors, each operating independently to decrease the immune response to immunization with RSV subunit vaccine, it seems unlikely that an RSV subunit vaccine will be able to induce the relatively high level of serum neutralizing antibodies (approximately 1:300) that is needed to protect the young infant from serious lower respiratory tract disease (Prince et al., 1985). These three factors alone would probably limit the usefulness of RSV subunit vaccines in infants. But, in addition, the humoral and cellular immune responses to immunization with the RSV subunit vaccines is very similar to those induced by the deleterious FI-RSV vaccine. This similarity suggests that disease potentiation in humans could be a consequence of immunization with RSV subunit vaccine, as it was with FI-RSV.

Other approaches

Synthetic peptide, anti-idiotype antibodies, and ISCOM formulated RSV vaccines are being evaluated as candidate vaccines (Palomo et al., 1990; Trudel et al.,

1991a; Trudel et al., 1991b; Trudel et al., 1992). Sufficient information on the safety, immunogenicity, and efficacy of these preparations is not available at this time to determine if they offer an advantage over the more extensively evaluated subunit vaccines. Furthermore, the phenomenon of immune-mediated disease potentiation means that any non-replicating, subunit-type vaccine must of necessity be developed with considerable caution.

Disease potentiation

Inactivated RSV vaccines are not currently being considered vaccine candidates, but recent observations in the context of the previous observations in the 1960's and 1970's have permitted the formulation of a plausible mechanism for the unexpected finding of disease potentiation seen in the FI–RSV vaccinees during natural RSV infection or reproduced in experimental animals receiving FI–RSV or subunit vaccines (Kapikian et al., 1969; Kim et al., 1969; Murphy et al., 1986b; Nicholas et al., 1990; Kim et al., 1976; Connors et al., 1992b). Two major factors are thought to have contributed to enhanced disease in FI–RSV vaccinees. First, FI–RSV failed to induce a high level of resistance to RSV replication (Kapikian et al., 1969; Kim et al., 1969). FI–RSV vaccinees developed high titers of serum antibodies to the F glycoprotein as determined by ELISA, but these antibodies had a low level of neutralizing activity (Murphy et al., 1986b). As outlined above, passive transfer of antibodies with low neutralizing activity failed to protect cotton rats from RSV infection, indicating that these antibodies had insufficient antiviral activity in vivo (Connors et al., 1992a). In addition, it is unlikely that appreciable secretory IgA was induced in the respiratory tract of the FI–RSV infant vaccinees because the FI–RSV vaccine was given parenterally. It is also unlikely that a $CD8^+$ CTL response developed in FI–RSV vaccinees, suggesting that the most potent antiviral effector cell of the T-cell component of the immune system was deficient in FI–RSV vaccinees (Nicholas et al., 1990; Connors et al., 1992b). Thus, with the local and systemic humoral immunity and cellular immunity to RSV being largely deficient in FI–RSV vaccinees, pulmonary RSV replication was not restricted in the vaccinees during subsequent natural infection. Pulmonary RSV replication then provided the stimulus for immune mediators to cause enhanced disease. The second factor postulated to be involved in the enhanced disease in the vaccinees was a state of heightened $CD4^+$ T-cell activity induced by immunization with FI–RSV. This was suggested previously by the increased lymphocyte proliferative response of peripheral blood lymphocytes of FI–RSV vaccinees to RSV antigens (Kim et al., 1976). Pulmonary infiltration that occurred following RSV challenge of FI–RSV-immunized mice was completely dependent on $CD4^+$ T-cells (Connors et al., 1992b). The type of priming of a mouse, be it by infection with live RSV or by immunization with inactivated or subunit vaccine, determines the pattern of T-helper cell subsets present in the lungs of mice challenged with RSV (Graham et al., 1993). Priming of mice with FI–RSV or F subunit vaccine induced a pattern of cytokine mRNA expression in lungs, suggesting a predominant Th_2-like lymphocytic response, whereas infection with RSV primed for a predominant Th_1 response. Thus, FI–RSV induced a qualitatively different pattern of

$CD4^+$ T-cell response than that induced by virus infection. We (Connors, Morse et al., unpublished observations) have recently found that depletion of IL4 and IL10, but not IL2 or gamma interferon, abrogates the pulmonary histopathology in FI–RSV immunized mice following RSV challenge. Since IL4 and IL10 are products of Th_2 cells, these depletion studies confirm and extend the results of cytokine mRNA patterns in vivo. Thus, the other imbalances in the immune response of FI–RSV immunized mice outlined above (IgG binding antibodies with low neutralizing activity, serum IgG antibodies without mucosal IgA antibodies, RSV specific $CD4^+$ T-cells without $CD8^+$ T-cells) were accompanied by yet another imbalance: an altered ratio of Th_1 to Th_2 T-cells present in the pulmonary inflammatory cell infiltrate. Considering these findings together, we suggest that in the FI–RSV infant vaccinees a heightened $CD4^+$ T-cell response occurred at the sites of RSV replication in the bronchioles and alveoli. The more severe bronchiolitis and pneumonia observed in the vaccinees infected with RSV reflected the more extensive $CD4^+$ T-cell-dependent infiltration of inflammatory cells into the bronchioles and alveoli occurring in pulmonary structures already damaged by replication of this cytopathic virus. It has recently been demonstrated that RSV-specific $CD4^+$ T-cells can augment disease in mice infected with RSV which clearly demonstrates that RSV-specific $CD4^+$ T-cells, like $CD8^+$ T-cells, can mediate severe pulmonary pathology (Alwan et al., 1992). Thus, we suggest that the enhanced disease was a $CD4^+$ T-cell-mediated pulmonary delayed-type hypersensitivity reaction. These observations with FI–RSV have implications for the development of RSV vaccines. It is reasonable to predict that in order to be useful in RSV immunoprophylaxis, immunogens will have to induce (1) both serum and mucosal antibodies with high neutralizing activities and (2) a pattern of cellular immune responses similar to that induced by infection with wild-type RSV and dissimilar from that induced by FI–RSV.

3. Live virus vaccines

Vectored vaccines

Vaccinia virus, a live attenuated virus vaccine previously used to prevent smallpox in humans, is being evaluated as a vector to carry foreign genes encoding protective viral antigens for the purpose of inducing protective immunity to the foreign virus. In addition to their possible use as a vaccine, vaccinia-recombinant viruses are very useful for the identification of the contribution of individual RSV proteins in resistance to RSV infection. Regardless of the type of RSV vaccine which will ultimately be used, it is important to know which viral components are responsible for protective immunity. The ability of nine vac-RSV recombinants expressing each of the known RSV proteins except L to induce resistance to RSV challenge in BALB recombinant mice homozygous for the $H\text{-}2^d$, $H\text{-}2^b$, or $H\text{-}2^k$ MHC haplotype was studied (Connors et al., 1991; Kulkarni et al., 1993). Vaccinia-F (vac-F) and vaccinia-G (vac-G) induced almost complete resistance to RSV challenge in mice of each of the three MHC haplotypes. Only one other vaccinia

recombinant (vac-M2) induced significant resistance, and this was evident only in BALB/c(H-2^d) mice. This M2-induced resistance was mediated by $CD8^+$ CTL, but the resistance was very transient and waned completely by 45 days after infection with vac-M2. An accelerated clearance of virus was not observed in mice challenged with RSV 45 days after immunization with vac-M2, indicating that if memory $CD8^+$ cells were present, they could not be activated from their resting state in time to alter the course of RSV replication in mice (Kulkarni et al., 1993). In contrast, the immunity induced by vac-F and vac-G was long-lived and mediated by antibodies (Connors et al., 1991). These results indicated that vectored vaccines need contain only the RSV antigens that induce neutralizing antibodies and that other RSV proteins that do not induce a long-lasting immunity in individuals of diverse MHC haplotypes need not be included in the vaccine.

The vac-RSV recombinant viruses administered parenterally were able to induce immunity in rodents (Johnson et al., 1987; Elango et al., 1986; Olmsted et al., 1986; Stott et al., 1987; Sullender et al., 1990; Wertz et al., 1987). This immunity resembled that conferred by passive antibodies in that the lower respiratory tract was resistant to replication of challenge RSV, but the upper respiratory tract remained susceptible. In contrast to subunit vaccine, antibodies induced by infection with vac-F and vac-G were able to passively protect naive cotton rats (Connors et al., 1992a). Thus, the vac-RSV recombinant viruses induced antibodies of high functional activity in vitro (neutralization) and in vivo (protection) and, therefore, the response to the vac-RSV recombinant vaccines was clearly distinguishable from that to the RSV subunit vaccines (Connors et al., 1992a). The immunity induced by vac-F and vac-G administered intradermally was readily suppressed by passive antibodies (Murphy et al., 1988a). However, the suppressive effects of passively acquired antibody on immunity induced by intradermally administered vac-RSV recombinants could be partially overcome by administration of the vaccinia recombinants by the intranasal route (Murphy et al., 1989a). Disease potentiation was not observed in vac-RSV immunized cotton rats following challenge with RSV (Connors et al., 1992a). Mice immunized parenterally with a vac-F recombinant did develop increased pulmonary histopathology upon RSV challenge compared to mice immunized with a control vaccinia virus (Stott et al., 1987), but there was no evidence that this was similar to the response associated with immunization of cotton rats with FI–RSV. Indeed, mice routinely develop pulmonary histopathology following second infection with RSV, and so the presence of a certain level of pulmonary histopathology in immunized mice following RSV challenge is to be expected (Connors et al., 1992b; Graham et al., 1991). However, unexpected pulmonary responses have been seen in mice immunized with vac-G, in which an increased number of eosinophils was present in the bronchioalveolar lavages of RSV-challenged mice, but not control mice (Openshaw et al., 1992). This clearly indicates that careful attention must be paid to possible adverse effects of immunization with vectored-RSV recombinant viruses.

Initial evaluation of vac-RSV recombinants in monkeys was encouraging in that owl monkeys immunized with vac-F and vac-G developed neutralizing antibodies and were resistant to challenge with RSV (Olmsted et al., 1988). However, similar

experiments in seronegative chimpanzees, our closest primate relative in which RSV infection resembles that in humans, revealed that intradermal immunization with vac-F and vac-G provided only low to moderate levels of serum neutralizing antibodies, inconsistent resistance in the lower respiratory tract, and no resistance in the upper respiratory tract (Collins et al., 1990b; Crowe et al., 1993). Interestingly, extremely high titers of neutralizing antibodies developed in the serum of the vac-F and vac-G immunized chimpanzees upon RSV challenge. In summary, vac-RSV recombinants induce only a marginal level of resistance in chimpanzees that likely would be further suppressed by maternally-derived antibodies. This weak immunogenicity combined with the possible virulence of vaccinia recombinants in immunosuppressed infants makes vac-RSV recombinants unattractive candidate vaccines. Vaccinia-PIV3 recombinants were also found to be immunogenic and efficacious in rodents and monkeys, but have not been evaluated further because of the concerns of using a vaccinia-based vector in infants, some of whom would be immunosuppressed due to perinatal human immunodeficiency virus infection (Spriggs et al., 1987; Spriggs et al., 1988). It is possible that more attenuated poxvirus vectors, such as avipox, NYVAC, or MVA, could be used as vectors of RSV or PIV3 antigens (Baxby and Paoletti, 1992; Sutter and Moss, 1992; Tartaglia et al., 1992). However, such recombinants would have to be more immunogenic than the vaccinia-RSV recombinants evaluated above, which were derived from the WR strain of vaccinia, and would have to be immunogenic and protective in the presence of maternal antibodies.

The results with vac-RSV recombinants were discouraging, but suggested that a vectored RSV vaccine given by a mucosal route might be immunogenic in the presence of maternal antibody (Murphy et al., 1989a; Collins et al., 1990a). The possible use of an adenovirus-RSV (ad-RSV) recombinant virus vaccine was initially very attractive because this vaccine could be given by the oral or intranasal route; in the latter case, this would protect the upper as well as the lower respiratory tract. This would be in contrast to the subunit vaccine and the intradermally administered vac-RSV recombinant viruses, neither of which protected the upper respiratory tract (Collins et al., 1990a). Initial studies in rodents and dogs demonstrated that ad-RSV recombinants were immunogenic and efficacious and could protect both the upper and the lower respiratory tracts (Collins et al., 1990a; Hsu et al., 1991; Hsu et al., 1992). However, the ad-RSV did not appear to be immunogenic in a chimpanzee, despite three sequential immunizations with ad-4, ad-5, or ad-7 RSV F recombinants. These adenovirus-based vectors appeared to suffer from the same disadvantage as vac-RSV recombinants, namely, poor immunogenicity in seronegative chimpanzees.

Live attenuated RSV vaccines

Live attenuated RSV vaccines appear to be the most attractive candidates for several reasons. First, live RSV vaccines would be anticipated to stimulate an immune response that resembles the response to wild-type virus infection, including the induction of serum and mucosal antibodies that are able to protect both the upper and lower respiratory tract, as well as the stimulation of a balanced

cellular immune response. Second, infection and immunization in the presence of maternal antibodies is possible, since wild-type RSV can infect and replicate efficiently in infants possessing substantial titers of residual maternally-acquired serum antibodies. Third, immunization of RSV-seronegative infants with live attenuated RSV vaccines was not associated with disease potentiation during subsequent natural RSV infection in the vaccinees (Chanock and Murphy, 1991).

How will a live attenuated virus vaccine induce resistance when wild-type infection itself, in some instances, fails to prevent serious illness during a second infection, especially considering that the immunity induced by live attenuated virus infection is likely to be weaker than that induced by wild-type virus infection? There are two answers to this important question. First, because a single dose of a live virus vaccine will not be sufficient to achieve a high level of durable immunity, the vaccine will need to be given several times during the first few months of life. Data in the literature suggest two infections with wild-type virus are needed to ensure a durable serum and local antibody response (Kaul et al., 1981; Wagner et al., 1989), and this is a partial explanation for the greatly decreased incidence of severe RSV disease during the third or subsequent RSV infection. A schedule of immunization like that for the live poliovirus vaccines may need to be followed, with the first dose of vaccine being given by 1 month of age. The optimal schedule will have to be determined by experimentation, but it might require two immunizations within the first two months of life. Fortunately, the immunization procedure will be trivial, involving the application of nose drops containing live vaccine virus. Second, since two antigenically distinct subgroups of RSV exist, the live RSV vaccine will likely be a bivalent vaccine. Reinfection with disease reflects not only waning immunity to the first RSV infection, but also antigenic diversity when the second infection virus is of the heterologous RSV subtype. Thus, multiple administrations of a bivalent RSV subgroup A and B vaccine during the first several years of life will be needed to induce a sustained level of serum and mucosal antibodies that will protect against severe RSV bronchiolitis and pneumonia in infancy and early childhood. As indicated above, it is possible that two doses might need to be given within the first few months of life. Repeated immunization with live RSV vaccine at the mucosal surface might even protect against rhinitis, pharyngitis, and otitis media experienced by RSV-infected infants and children.

Three types of live attenuated RSV vaccines have been evaluated: (1) host range mutants; (2) cold-passaged mutants; (3) and temperature-sensitive (*ts*) mutants. The experience with these three vaccines has been recently reviewed and will not be detailed here (Chanock and Murphy, 1991), but only those approaches not discussed in that summary will be briefly presented.

Bovine RSV (BRSV) has been considered as a live attenuated RSV vaccine to protect humans against human RSV (HRSV) (Prince et al., 1991; Piazza et al., 1993). Infection of rodents and owl monkeys with BRSV protected against HRSV challenge. However, infection of chimpanzees with BRSV failed to induce resistance to infection or illness upon challenge with HRSV (R.M. Chanock, unpublished observations). The findings with BRSV, therefore, resemble those with subunit and vectored RSV vaccines in that it is relatively easy to protect rodents

and monkeys which are only semi-permissive for RSV infection, but it is more difficult to protect the seronegative chimpanzee, a fully permissive host. It appears unlikely at this juncture that the distantly related (Lerch et al., 1990; Lerch et al., 1991) BRSV virus will be able to protect humans against HRSV.

A second approach is being pursued by Dr. Valerie Randolph and her colleagues of Lederle-Praxis Biologicals (personal communication). Two wild-type RSV strains, designated RSV3A and RSV2B (subgroup A and B, respectively) that had been isolated from infected pediatric patients in primary rhesus monkey kidney cells were biologically cloned and passaged up to 42 times in Vero cells using three different cold-adaptation strategies. The strategies differed in the rate at which cold-adaptation was attempted by varying the temperature and the length of time the virus was maintained at each temperature. For RSV2B, each of the cold-adaptation strategies yielded progeny virus able to replicate more efficiently at 20°C than wild-type virus. In contrast, RSV3A could only be adapted to growth at 20°C using a strategy of gradual adaptation at successively lower temperatures. Four RSV2B (p33F, p24G, p20L, and p34L) and three RSV3A (p20E, p20F, and p28F) mutants were selected from a panel of *ts* clones derived by plaque selection of cold-adapted (*ca*) virus. Each of the mutants was *ts* at 39°C and possessed the *ca* phenotype, i.e., more efficient replication at 20°C than that observed with wild-type virus. Each of the four RSV2B mutants was restricted in replication in cotton rats and the RSV2B p20L mutant was attenuated in seropositive chimpanzees. The *ca ts* mutants are currently being studied further in animals.

The immunogenicity and pathogenicity of *ts* mutants of the RSS-2 strain of RSV are being evaluated in human volunteers (Pringle et al., 1991; Pringle et al., 1993). Two viruses, *ts*1A and *ts*19A, were further mutagenized to provide a series of viruses with different levels of temperature sensitivity of plaque formation in vitro, namely, *ts*19A (unable to form plaques at 39°C), *ts*19B (38°C), *ts*1A (39°C), *ts*1B (38°C), and *ts*1C (37°C). The *ts*1C virus was derived by mutagenesis of *ts*1B. Each of these viruses retained the ability to infect seropositive adult volunteers and to induce a serological response. However, each appeared to retain the ability to induce symptoms in the upper respiratory tract of adults, even though the frequency and severity of the illness was less than that caused by infection with wild-type RSS-2 virus. The illness observed in adults with the *ts*1C virus, i.e., the most defective strain in vitro, was not accompanied by evidence of infection with vaccine virus, suggesting that the illness was not caused by the vaccine virus. Since viruses that have residual reactogenicity in seropositive adults are likely to be incompletely attenuated for seronegative infants and children, the *ts*1A and *ts*1B live attenuated candidate vaccines might be insufficiently attenuated for the target population (Chanock and Murphy, 1991). However, further study of the *ts*1C virus in infants and children is needed to determine if it is satisfactorily attenuated and immunogenic in the target population.

The Laboratory of Infectious Diseases, National Institutes of Allergy and Infectious Diseases, NIH (USA) and Wyeth-Ayerst Research have initiated a cooperative project to produce a live attenuated bivalent RSV vaccine. The project was initiated by evaluation of the level of attenuation, genetic stability, and efficacy

of three *ts* mutants of the RSV A2 (subgroup A) wild-type virus, designated *ts*-1, *ts*-1-NG1, and *ts*-4, in seronegative chimpanzees (Crowe et al., 1993). These mutants had been prepared previously by one (*ts*-1, *ts*-4) or two (*ts*-1, NG1) rounds of mutagenesis (Chanock and Murphy, 1991). Each of the *ts* mutants was highly restricted in replication in the lower respiratory tract of the chimpanzee, but still retained the capacity to induce significant rhinorrhea. Each of the three *ts* mutants underwent partial reversion to a non-*ts* (ts^+) phenotype during replication in a minority of the chimpanzees. The ts^+ virus present in the upper respiratory tract of the chimpanzees did not spread to the lower respiratory tract and represented only a small fraction of the virus present in the nasopharyngeal swab specimens. The ts^+ virus that was detected did not outgrow or displace the *ts* virus present in the animal. The *ts* mutants were highly immunogenic and provided resistance that effectively restricted RSV replication following virus challenge. In contrast, vac-RSV recombinants tested in parallel were less immunogenic: they provided partial protection in the lungs of two of four chimpanzees challenged with RSV, but failed to protect the upper respiratory tract. The seronegative chimpanzee will serve as a model for the rapid evaluation of further attenuated live RSV vaccines.

A cold-passaged RSV mutant, designated *cp*-RSV, which acquired host range mutations during 52 passages at low temperature in bovine tissue culture, was completely attenuated for seropositive adults and children but retained the capacity to cause upper respiratory disease in seronegative infants (Friedewald et al., 1968). Additional attenuating mutations, such as temperature-sensitive (*ts*) and small-plaque (*sp*) mutations, were introduced into the *cp*-RSV, which is a ts^+ virus, in order to generate a derivative which would be satisfactorily attenuated in seronegative infants and young children (Crowe et al., 1994b). Nine mutants of *cp*-RSV, which had acquired either the *ts* or small-plaque *sp* phenotype, were generated by chemical mutagenesis with 5-fluorouracil. The two *ts* mutants with the lowest in vitro shut-off temperature, namely the *cpts*-248 (38°C) and *cpts*-530 (39°C) mutants, were the most restricted of the nine *cp*-RSV mutant progeny in replication in Balb/c mice. In seronegative chimpanzees, the *cpts*-248 mutant replicated 4-fold less efficiently in the nasopharynx and caused significantly less rhinorrhea than its *cp*-RSV parent. The *cpts*-248 mutant virus, like its *cp*-RSV parent, was 1000-fold restricted in replication in the trachea compared to wild-type RSV. Previously, another candidate RSV live attenuated vaccine strain, a mutant designated *ts*-1, exhibited some instability of its *ts* phenotype following replication in susceptible humans or chimpanzees. Hence, *ts* progeny of *cp*-RSV were sought that exhibited a greater degree of stability of the *ts* phenotype than the prototype *ts*-1 mutant. The *cpts*-248 and *cpts*-530-progeny viruses exhibited a greater degree of stability of the *ts* phenotype in nude mice than the *ts*-1 virus, and in chimpanzees, the former mutant also exhibited a greater stability of its *ts* phenotype than *ts*-1. The *cpts*-248 mutant was immunogenic and induced a high level of resistance in chimpanzees to subsequent challenge with wild-type RSV. The *cpts*-248 mutant therefore exhibits a set of properties which make it a promising vaccine candidate. These desirable properties of *cpts*-248 suggest that the mutant should be tested in humans for its suitability in immunoprophylaxis.

Since the *cpts*-248 virus might not be sufficiently attenuated for fully susceptible seronegative human infants and young children, more attenuated derivatives of this virus were sought by subjecting it to a second round of mutagenesis. Additional attenuating mutations such as *sp* and *ts* mutations were introduced into RSV *cpts*-248 by chemical mutagenesis with 5-fluorouracil with the intent of obtaining *cpts*-248 derivatives that are more attenuated in mice or chimpanzees and that are more genetically stable following replication in vivo. Ten mutants of RSV *cpts*-248 which had acquired a *sp* phenotype or a second *ts* mutation were generated by chemical mutagenesis. Five *cpts*-248 derivatives which had acquired mutations that specified a 36°C shut-off temperature for plaque formation and one which had acquired only a *sp* phenotype were more restricted in replication in Balb/c mice than the *cpts*-248 parental strain. One mutant, designated RSV *cpts*-248/404 (shutoff temperature 36°C), was 100 times more restricted in replication in the nasal turbinates of mice and 1000 times more restricted in the nasopharynx of seronegative chimpanzees than its *cpts*-248 parent. The *cpts*-248/404 mutant was completely restricted in replication in the lower respiratory tract of chimpanzees even following direct intratracheal administration. The *ts* phenotype of the *cpts*-248/404 mutant was stable during replication in vivo in mice and chimpanzees. Chimpanzees immunized with *cpts*-248/404 were fully protected against upper respiratory tract disease and lower respiratory tract virus replication upon subsequent challenge with wild-type virus. The *cpts*-248/404 virus and related mutants exhibit many desirable characteristics which make them promising vaccine candidates (Crowe et al., 1994a).

4. Vaccine development for PIV3

General considerations

Parainfluenza virus type 3, also a member of the Paramyxoviridae family, causes croup, bronchiolitis, and pneumonia, and is responsible for about 11% of hospitalizations for pediatric respiratory tract disease (Murphy et al., 1988b; Chanock and McIntosh, 1990). It differs from RSV in that the peak incidence of severe disease is not seen at two months of age, but occurs somewhat later within the first two years of life. However, severe disease can occur within the first six months of life and immunization should be initiated before six months of age. The protective antigens of PIV3 are the hemagglutinin-neuraminidase (HN) glycoprotein (the attachment protein) and the fusion (F) glycoprotein, both of which induce neutralizing antibodies (Chanock and McIntosh, 1990). Protection against PIV3 in humans will likely require the induction of both humoral and mucosal antibodies active against these proteins (Chanock and McIntosh, 1990). Previously-tested, inactivated parainfluenza virus vaccines were not sufficiently immunogenic to be protective in humans (Chin et al., 1969), but disease potentiation was not observed in these studies. However, since this phenomenon was seen for two other members of the Paramyxoviridae family (RSV and measles), the possibility exists that further study will find that disease potentiation can occur with inactivated PIV3 or with

PIV3 subunit vaccines that induce a similar immune response. The extensive safety record of live attenuated measles and mumps virus vaccines, together with our experience with experimental live RSV vaccines, suggests that it is unlikely that immunization with live attenuated PIV3 virus vaccines would be followed by disease potentiation.

PIV3 subunit vaccines

Several recent approaches to the production and purification of HN and F glycoproteins of PIV3 for use in a subunit vaccine have been explored (Ambrose et al., 1991; Ewasyshyn et al., 1992; Hall et al., 1991; Ray et al., 1988; Van Wyke Coelingh et al., 1987). One of these approaches, which is still being actively pursued, employs as the subunit vaccine the HN and F glycoproteins of PIV3 which were isolated from purified virus by solubilization with Triton X-100 and purified over a lentil lectin-affinity column using methyl-α-D-mannopyranoside for elution (Ambrose et al., 1991). Immunization with the HN and F subunit vaccine induced functional antibodies, including those with neutralizing, hemagglutination-inhibiting, and fusion-inhibiting activities. Cotton rats immunized parenterally with the HN and F subunit vaccine and challenged intranasally with PIV3 were protected in both the upper and lower respiratory tract. Disease potentiation was not seen in the immunized, PIV3-challenged animals, and resistance to virus replication in the lung correlated with decreased pathology compared to that observed in PIV3-challenged, unimmunized control animals. These preliminary results are encouraging and further evaluation of this candidate vaccine is continuing. Chimeric subunit vaccines have been produced that consist of the PIV3 F ectodomain linked in frame to that of PIV3 HN. The F HN subunit vaccine was immunogenic in cotton rats and exhibited greater efficacy than combined immunization with purified F and HN glycoproteins in cotton rats (Lehman et al., 1993; Brideau et al., 1993). In addition, a chimeric virus consisting of the RSV F linked to the PIV3 HN has been produced that can induce resistance to both viruses in rodents (Homa et al., 1993).

PIV3 microencapsulated whole virus vaccines

Purified PIV3 has been microencapsulated in biocompatible and biodegradable DL-lactide and glycolide copolymer and administered intraperitoneally (i.p.), orally, or intranasally to hamsters (Ray et al., 1993). Following PIV3 challenge, only hamsters immunized i.p. were protected.

5. Live virus vaccines

Bovine PIV3 (BPIV3)

BPIV3 was chosen as a candidate live-virus vaccine to protect against infection with HPIV3 for several reasons. First, antigenic analysis using monoclonal antibodies and post-infection sera from rodents and primates indicated that the surface glycoproteins of the BPIV3 and HPIV3 are about 25% related by cross-neutraliza-

tion (Van Wyke Coelingh et al., 1988). An examination of the antigenic site-specific serum antibody responses of rhesus monkeys and chimpanzees infected with BPIV3 to the HN and F glycoproteins of HPIV3 revealed that the majority of the sites recognized by humans undergoing infection with HPIV3 were also recognized by the post-BPIV3-infection sera of the monkeys and chimpanzees (Van Wyke Coelingh et al., 1990). This high degree of relatedness revealed by antigenic analysis was confirmed by sequence analysis of BPIV3 and HPIV3 HN and F glycoproteins which were found to be 77% and 80% related, respectively (Suzu et al., 1987). Second, infection of cotton rats or monkeys with BPIV3 induced resistance to subsequent challenge infection with HPIV3 (Van Wyke Coelingh et al., 1988). It was felt that BPIV3, which had evolved over a long period of time in its natural host, should possess sequences divergent from HPIV3 that restrict its replication and its virulence in humans. This mechanism of attenuation based on host range is analogous to that of cowpox (vaccinia) virus, which was much less virulent in humans than variola virus but, nonetheless, provided excellent protection against smallpox. This approach to immunization against viral pathogens has been termed the "Jennerian" approach. The replication of two different strains of BPIV3 was restricted 100- to 1000-fold in Old World primates (i.e., rhesus monkeys or chimpanzees), but was sufficient to induce high levels of serum neutralizing antibodies to HPIV3 (Van Wyke Coelingh et al., 1988). The combined properties of restriction of replication and induction of a protective immune response to HPIV3 in non-human primates makes the BPIV3 a promising candidate for use as a live virus vaccine.

Initial studies of BPIV3 in humans were performed in seropositive adults in whom BPIV3, like the HPIV3 wild-type virus studied concurrently, was avirulent and poorly infectious (Clements et al., 1991). Studies of the BPIV3 in seropositive and seronegative children are currently being performed. The BPIV3 was restricted in replication, poorly infectious, and avirulent in both seropositive children and adults (Karron, R. and P. Wright, unpublished observations). In contrast, the BPIV3 was highly infectious for seronegative vaccinees when administered at a dose of $10^{4.0}$ or $10^{5.0}$ $TCID_{50}$, with most vaccinees shedding virus or developing a serum antibody response. The virus appeared to be non-reactogenic at doses of $10^{3.0}$ to $10^{5.0}$ $TCID_{50}$. These initial studies were encouraging, and further evaluation of infectivity, immunogenicity, transmissibility, and genetic stability are currently underway.

A second live attenuated virus is being developed. A human wild type strain of PIV3, strain JS, was cultivated in primary monkey kidney tissue culture at low temperatures for 45 passages and mutants were selected after 12, 18, or 45 passages and designated cp12, cp18, or cp45, according to their cold-passage level (Belshe and Hisson, 1982). Three phenotypic markers were acquired during the process of low temperature passage: cold-adaptation, i.e., the ability to replicate efficiently at 20°C; temperature sensitivity, i.e., restriction of growth at 40°C in tissue culture; and attenuation (*att*), manifested by restriction of replication compared to wild-type virus in hamsters (Belshe and Hisson, 1982; Crookshanks and Belshe, 1984). The cpPIV3 mutants protected hamsters against PIV3 chal-

lenge (Crookshanks-Newman and Belshe, 1986). Each of the three cp mutant viruses was attenuated in seronegative rhesus monkeys (Hall et al., 1992). The level of temperature-sensitivity of the cp mutants directly correlated with the degree of attenuation in monkeys, with the cp12 being the least *ts* and the least attenuated, whereas the cp45 mutant was the most *ts* and the most attenuated. Data derived from the evaluation of the cp12, cp18, and cp45 viruses in rhesus monkeys indicated that cp45 appeared to be the most promising of the three cp mutants because it was the most restricted in pulmonary virus replication and its *ts* phenotype was the most stable following replication in vivo. The cp45 mutant was also the only one of the three cold-passaged viruses that possessed both *ts* and non-*ts* mutations that contributed to the attenuation phenotype in rhesus monkeys, suggesting that the cp45 virus had acquired a greater number of attenuating mutations compared to the cp12 and cp18 mutants. The multiple genetic mutations of the cp45 virus could result in enhanced stability of the attenuation phenotype after replication in vivo. The nucleotide sequence of cp12 and cp45 viruses has been completed and compared to that of their JS wild-type parent virus (Stokes et al., 1993). Eight nucleotide changes in non-coding or coding regions were identified in each attenuated virus that were considered mutations that could contribute to the in vitro or in vivo phenotype of these two viruses.

The cp45 mutant was also evaluated for safety, immunogenicity, genetic stability, and protective efficacy in seronegative chimpanzees (Hall et al., 1993). Previous studies of cp12 indicated that the virus was only partially attenuated in chimpanzees (Clements et al., 1991). The cp45 virus was highly restricted in replication in both the upper and lower respiratory tract of the chimpanzee compared to the JS wild-type parent virus. Animals immunized with cp45 virus were also highly resistant to wild-type PIV3 challenge. The cp45 virus present in the respiratory tract secretions of chimpanzees retained the *ts* phenotype, but some loss of the level of temperature sensitivity was observed in the virus isolated in tissue culture (referred to as isolates), i.e., the input cp45 was more restricted in plaque formation at 38°C than the cp45 isolates, but all the isolates were *ts* at 40°C. Stability of the attenuation phenotype was demonstrated by the administration of an isolate of cp45 (*ts* at 39°C), obtained after ten days of replication in a chimpanzee, to two additional chimpanzees. This chimpanzee-passaged cp45 virus was attenuated in both the upper and lower respiratory tracts, indicating that the attenuation phenotype of the cp45 virus was stable following replication in chimpanzees, despite the partial loss of the *ts* phenotype. Indeed, other studies indicate that major elements of the *att* phenotype of the cp45 virus do not involve temperature sensitivity (Hall et al., 1992). These results provide a basis on which to proceed to clinical trials in humans.

The cp12, cp18, and JS wild-type viruses were poorly infectious in seropositive human adults. Because of the limited attenuation and genetic stability of the cp12 virus in chimpanzees, it was not studied further in children. The cp18 virus was evaluated for safety, immunogenicity, transmissibility, and genetic stability in a double-blind, placebo-controlled study of 95 infants and young children (Belshe et al., 1992). None of 19 seropositive children 41 to 124 months old became infected

when $10^{6.0}$ $TCID_{50}$ of vaccine virus was administered intranasally. Two of nine and seven of twenty-four younger seropositive children given 10^5 or 10^6 $TCID_{50}$ of cp18, respectively, became infected. Each of four seronegative young children given $10^{6.0}$ $TCID_{50}$ of cp18 virus became infected. Illness was not observed in seropositive children, but two of the four seronegative children developed a mild afebrile illness characterized by rhinorrhea and wheezing on auscultation. In one case, cp18 vaccine virus spread from a vaccinee to an unvaccinated sibling but did not cause illness. The incomplete attenuation and ready transmissibility of the cp18 virus makes this an undesirable PIV3 candidate vaccine. Therefore, the more attenuated and stable cp45 mutant is being evaluated currently in seropositive and seronegative young infants and children to examine its usefulness as a vaccine.

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Cold-passaged, temperature-sensitive mutants of human respiratory syncytial virus (RSV) are highly attenuated, immunogenic, and protective in seronegative chimpanzees, even when RSV antibodies are infused shortly before immunization

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*A cold-passaged (*cp*) temperature-sensitive (*ts*) RSV mutant, designated RSV* cpts*-530, which possesses host-range mutations acquired during 52 passages at low temperature in bovine tissue culture and one or more* ts *mutations induced by chemical mutagenesis (shut-off temperature 39°C) was found previously to be tenfold restricted in its replication in mice as compared to wild-type virus and stable genetically in nude mice. In the current study, we introduced additional attenuating mutations, such as small-plaque (*sp*) or* ts *mutations, into* cpts*-530 by chemical mutagenesis with 5-fluorouracil, with the intent of obtaining derivatives of* cpts*-530 that were more attenuated in mice or chimpanzees and that were more stable genetically following replication* in vivo. *Fourteen mutants of RSV* cpts*-530 which had acquired an additional* ts *mutation were identified and found to be more restricted in replication in BALB/c mice than the* cpts*-530 parental strain. One mutant, designated* cpts*-530/1009 (shut-off temperature 36°C), was 30 times more restricted in replication in the nasal turbinates of mice and threefold more restricted in the nasopharynx of seronegative chimpanzees than its* cpts*-530 parent. Like its parent, this mutant was highly restricted (30 000-fold) in replication in the lower respiratory tract of chimpanzees even following direct intratracheal inoculation. The* cpts*-530 and* cpts*-530/1009 mutants exhibited a high level of stability of the* ts *phenotype during replication in chimpanzees.*

The immunogenicity and protective efficacy of the cpts*-530/1009 mutant and that of two other previously described candidate live attenuated RSV vaccines were compared in seronegative chimpanzees, some of whom were pretreated with RSV immune globulin by the intravenous route to simulate the condition of the very young infant who possesses passively acquired maternal antibodies. The three candidate vaccine strains were immunogenic and induced significant resistance to RSV challenge in both groups of chimpanzees. Interestingly, the chimpanzees infused with RSV antibodies prior to immunization were primed more effectively for an unusually high serum neutralizing antibody response to infection with challenge virus than chimpanzees which did not receive such antibodies. This high level booster response occurred despite marked restriction of replication of the challenge virus. Thus, the* cpts*-530/1009 virus and related mutants exhibit many desirable characteristics which make them promising vaccine candidates.*

Keywords: Respiratory syncytial virus; passive antibody transfer vaccines; attenuated; chimpanzee

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Respiratory syncytial virus (RSV) is the most common cause of serious viral lower respiratory tract disease in infants and children[1]. The peak incidence of severe lower respiratory tract disease requiring hospitalizati n caused by this virus occurs at the age of 2 months[2], about the time when the serum level of passively acquired maternal RSV neutralizing antibodies declines in some infants below levels associated with protection[2,3]. We have recently renewed our efforts to generate safe and effective live attenuated RSV vaccines for topical administration. Previous work has identified a number of significant obstacles to the successful development of live RSV mutants as vaccines. First, it has been difficult to produce live attenuated vaccines that achieve an acceptable balance between attenuation and immunogenicity. This is especially difficult for RSV because a successful vaccine must be satisfactorily attenuated and immunogenic for seronegative children greater than six months of age as well as for very young infants who possess varying levels, often quite high, of maternally derived serum RSV antibodies. The second major obstacle has been the instability of the *ts* phenotype, a laboratory marker of attenuation of vaccine virus, following virus replication in humans. In an attempt to overcome these two obstacles, we have used a strategy that involves the development of candidate vaccines that possess at least two different classes of attenuating mutations, e.g. host-range and *ts* attenuating mutations.

In a previous study chemical mutagenesis was used to introduce one or more *ts* mutations into a cold-passaged virus (*cp*-RSV) that had been partially attenuated previously by the acquisition of host-range mutations[4]. We describe here work in which the *cpts*-530 mutant was characterized *in vivo*, then further attenuated by introduction of a third attenuating mutation. It was hoped that the addition of two *ts* mutations would satisfactorily attenuate the *cp*-RSV (host-range) mutant and make it acceptable for immunization of fully susceptible, seronegative older infants, and that the combination of the different classes of mutations would increase genetic stability. Derivatives of *cpts*-530 exhibiting a wide range of attenuation were isolated and characterized *in vitro* and *in vivo*. Importantly, the present study extends our previous findings by comparing the immunogenicity of candidate RSV vaccines in: (1) seronegative chimpanzees, the most relevant experimental surrogate for the older seronegative infant, and (2) similar chimpanzees which possess passively acquired RSV antibodies. The latter chimpanzees simulate the immunological status of the very young infant who possesses transplacentally acquired maternal RSV antibodies. Live RSV vaccine candidates representing a wide spectrum of attenuation in chimpanzees were identified and each was shown to induce resistance to RSV challenge in both groups of chimpanzees.

METHODS AND MATERIALS

Cell culture

Vero cells, a continu us line of African green monkey kidney cells were obtained from the World Health Organization (W.H.O.) via the American Type Culture Collection (A.T.C.C.), Rockville, MD, at passage 134. Cells banked at passage 138 were recertified by Dr Louis Potash of P.R.I./Dyncorp (Rockville, MD) to be free of adventitious agents, and were maintained as previously described[4]. Vero cells were not used beyond passage 150. HEp-2 cells, a human epithelial transformed cell line obtained from the A.T.C.C. at passage 364, were maintained as previously described[4]. HEp-2 cells were not used beyond passage 400. HEp-2 cell monolayer cultures grown on 24-well tissue-culture plates (Costar) were used for all virus assays as previously described[5].

Viruses

The isolation and characterization of the wild-type RSV strain A2 and mutants derived from it, *cp*-RSV, *cpts*-248, *cpts*-248/404, and *cpts*-530, were described previously[4,6-8]. Fourteen mutants of *cpts*-530 were derived by chemical mutagenesis using 5-fluorouracil (5-FU, Sigma Chemicals) in Vero cell monolayer cultures using methods described previously[4,8]. Plaque progeny that produced small plaques at 32°C or that exhibited a decrease in plaque titer or size at 37°C in HEp-2 cell monolayer cultures were evaluated further. Fourteen mutants with one or both of these phenotypes were cloned by three plaque-to-plaque passages in Vero cells and subsequently amplified in Vero cells. The suspensions of the *cpts*-530 and *cpts*-530/1009 mutants that were used for experimental infection of chimpanzees contained virus amplified twice in Vero cells following plaque purification. Because infection of BALB/c mice with a large quantity of wild-type RSV ($\sim 10^{6.3}$ p.f.u. in a 0.1 ml inoculum) is required in order to recover $10^{5.0}$–$10^{6.0}$ p.f.u. virus per gram of lung or nasal turbinate tissue, concentrated suspensions of RSV A2, *cp*-RSV, *cpts*-248, *cpts*-248/404, *cpts*-530 and each of the fourteen *cpts*-530 mutants to be studied were prepared as previously described[8].

Virus characterization

Virus present in tissue culture harvests, lung or nasal turbinate homogenates of mice, or the nasopharyngeal or tracheal lavage fluids collected from experimentally infected chimpanzees were characterized as to *ts* phenotype by determining the efficiency of plaque formation (E.O.P.) on HEp-2 cell monolayer cultures maintained under a semisolid overlay at various temperatures as described previously[9]. Detection of plaques was facilitated by staining monolayers using an immunoperoxidase procedure as described previously[10]. Clindamycin ($100\ \mu g\ ml^{-1}$) and ciprofloxacin ($100\ \mu g\ ml^{-1}$) were added to the overlay of cultures used to quantitate virus recovered from chimpanzees.

Immunological studies

Serum RSV neutralizing antibodies were quantitated by a complement-enhanced 60% plaque reduction neutralization assay using RSV strain A2 and HEp-2 monolayer cultures[11] that were stained by an immunoperoxidase procedure as previously described[10]. Serum IgG antibodies binding to RSV F or G glycoprotein were quantitated in an ELISA using F or G glycoprotein that had been immunoaffinity-purified from RSV subgroup A (Long strain) infected cell lysates as described[12,13].

Animals

Respiratory-pathogen-free BALB/c mice were obtained from the National Cancer Institute (Frederick, MD) and used at 32 weeks of age. Mice were maintained in microisolator cages throughout the study. Mice in groups of six were inoculated intranasally under light methoxyflurane anesthesia on day 0 with $10^{6.3}$ p.f.u. of mutant or wild-type RSV in a 0.1 ml inoculum. Four days following inoculation, mice were sacrificed by CO_2 asphyxiation, and nasal turbinates and lung tissues were obtained separately for quantitation of virus and for characterization of recovered virus[5,10].

Thirty young male or female chimpanzees (*Pan troglodytes*) weighing 5.9–20.1 kg were pair-housed in large glass isolator suites and maintained as described previously[14]. The animals were on loan from Yerkes Regional Primate Center, Atlanta, GA; Southwest Foundation, San Antonio, TX; or the New Mexico Regional Primate Research Laboratory, Holloman AFB, NM. These chimpanzees lacked detectable serum neutralizing antibodies for RSV (titer <1:10). Groups of four seronegative chimpanzees were infected with either *cpts*-530/1009, *cpts*-530, *cp*-RSV, wild-type RSV strain A2, *cpts*-248, or *cpts*-248/404. Chimpanzees were inoculated either by both the intranasal (IN) and intratracheal (IT) routes with a dose of 10^4 p.f.u. in a 1.0 ml inoculum at each site, or by the IN route alone with a dose of 10^4 p.f.u. in a 1.0 ml inoculum, as indicated in the tables. Animals which received *cp*-RSV, RSV strain A2, *cpts*-248/404, or *cpts*-248, were described previously[4,8,9] and are included here for the purpose of comparison. The comparability of the studies was insured by the use of identical protocols and challenge virus suspensions. In addition, inoculation, sampling, and clinical scoring procedures were performed by the same individuals in each study. Additional groups of two seronegative chimpanzees were administered 100 mg kg^{-1} human RSV immune globulin[15] (Massachusetts Public Health Biologic Laboratories Lot No. RSV-5-50) by the intravenous route two days prior to immunization with *cpts*-248/404, *cpts*-248, or *cpts*-530/1009. This lot of intravenous immune globulin, comprising a purified IgG suspension derived from blood donations of volunteers with high serum RSV neutralizing antibody titers, was demonstrated to have a neutralizing antibody titer of 1:20 000 against RSV A2 as measured in the plaque reduction assay. The 100 mg kg^{-1} dose of this lot of immune globulin was selected with the intention of achieving a serum neutralizing antibody level in the chimpanzee similar to that of the normal 2-month-old human infant possessing maternally derived RSV antibodies. Following inoculation of virus, nasopharyngeal (NP) swab specimens were collected under ketamine anesthesia for quantitation of the amount of virus shed on days 1 through 10, 13, 16, and 20, and tracheal lavage specimens were collected on days 2, 4, 6, 8, 10, 13, 16, and 20 as described previously[9]. The amount of rhinorrhea was estimated daily and assigned a score by an experienced observer of 0–4 (0=none, 1=trace, 2=mild, 3=moderate, 4=severe).

Twenty-eight days following immunization, two of the seronegative chimpanzees immunized with each mutant were challenged IN and IT with 10^4 p.f.u. of wild-type RSV A2 in a 1.0 ml inoculum at each site. The six animals infused with antibodies just prior to immunization with one of the mutants were challenged 42 days following immunization (allowing two half-lives of serum IgG to elapse from the time of infusion to the time of challenge) in order that any protection observed against wild-type virus challenge might better represent immunity conferred by immunization rather than the residual effect of infused antibodies. NP swab specimens were collected every day, and tracheal lavage specimens were collected every other day for 10 days, in order to estimate quantity of virus shed. Rhinorrhea scores also were recorded daily. The response of these 16 RSV-challenged animals was compared with that of the four unimmunized control animals infected with RSV A2.

RESULTS

In vitro characterization of *ts* mutants

The *cpts*-530 mutant was mutagenized with 5-FU and 1325 plaque progeny were characterized. Fourteen plaque progeny were more *ts* than the *cpts*-530 parent as determined by E.O.P., and in addition, one of these exhibited the small-plaque (*sp*) phenotype (<50% wild-type plaque size at 32°C) (*Table 1*). The E.O.P. at different temperatures of the *ts* mutants of *cpts*-530 was examined in detail (*Table 1*) because previous studies demonstrated a correlation between the level of attenuation of a *ts* mutant and its degree of temperature-sensitivity *in vitro*[16]. The plaque titer of the wild-type RSV strain A2 or *cp*-RSV was not significantly reduced at 40°C. The shut-off temperature, i.e. the lowest temperature at which plaque titer was reduced at least 100-fold relative to the titer at permissive temperature (32°C), was 39°C for the *cpts*-530 parent and varied for the derivatives of *cpts*-530, ranging from ≤34 to 37°C (*Table 1*). Thus, the mutants derived from *cpts*-530 exhibited a wide spectrum of temperature-sensitivity.

Level of replication of *cpts*-530 mutants in BALB/c mice

The level of replication of the wild-type virus, *cpts*-530, and *ts* mutants of *cpts*-530 was compared in the nasal turbinates or lungs of BALB/c mice following IN inoculation of $10^{6.3}$ p.f.u. (*Table 1*). Because derivatives of another live attenuated mutant, the RSV A2 *cpts*-248 virus, had been extensively characterized previously in rodents and chimpanzees, we included for comparative purposes in this study two mutants from that lineage, namely *cpts*-248 and *cpts*-248/404 (representing mutants with high or low *in vivo* replicative capacity, respectively). The *cpts*-530 mutant (39°C shut-off temperature) exhibited a 30- or 40-fold reduction in replication in the lungs or nasal turbinates, respectively, compared with wild-type. The derivatives of *cpts*-530 were more restricted in replication than their partially attenuated parent in both nasal turbinates and lungs, with the small-plaque *cpts*-530/188 mutant derivative being the most restricted.

Level of attenuation, genetic stability, and efficacy of *cpts*-530 and *cpts*-530/1009 in seronegative chimpanzees

The *cpts*-530/1009 mutant was chosen for further evaluation in chimpanzees because it represented a virus with an intermediate level of replicative capacity in mice,

Table 1 The efficiency of plaque formation and level of replication in mice of 14 mutants derived from RSV *cpts*-530, compared with controls

RSV	*In vitro* efficiency of plaque formation: The titer of virus (log_{10} p.f.u. ml^{-1}) at the indicated temperature (°C)								Shut-off temperature (°C)[c]	Replication in mice[a] (mean log_{10} p.f.u. g^{-1} tissue of six animals ± s.e.m.)	
	32	34	35	36	37	38	39	40		Nasal turbinates	Lungs
A2 w.t.	6.3	6.3	6.1	6.2	6.3	6.3	6.1	5.6	>40	5.0±0.14	5.8±0.05
cp-RSV	6.5	6.2	6.2	6.2	6.1	6.0	6.1	5.6	>40	n.d.	n.d.
cpts-248	6.3	6.3	6.3	6.3	**3.7**[b]	<0.7	<0.7	<0.7	37	4.1±0.08	5.1±0.13
248/404	6.3	5.7[a]	4.3[b]	<0.7	<0.7	<0.7	<0.7	<0.7	35	2.1±0.19	3.6±0.10
cpts-530	6.2	6.3	6.2	6.1	**6.2**[a]	**5.5**[b]	<0.7	<0.7	39	3.4±0.09	4.3±0.14
530/346	5.9	5.9	5.7	4.7	**3.5**	<0.7	<0.7	<0.7	37	3.3±0.11	4.7±0.09
530/977	5.0	4.4	3.6	3.4	**2.8**[a]	<0.7	<0.7	<0.7	37	3.4±0.11	2.7±0.05
530/9	6.0	5.6	5.0	**3.5**[a]	**3.5**[b]	<0.7	<0.7	<0.7	36	2.1±0.06	3.5±0.08
530/1009	4.8	4.0	**3.7**[a]	**2.0**[b]	**1.5**[b]	<0.7	<0.7	<0.7	36	2.2±0.15	3.5±0.13
530/667	5.5	4.9	**4.5**[a]	**2.0**[b]	**0.7**[b]	<0.7	<0.7	<0.7	36	2.4±0.12	2.9±0.15
530/1178	5.7	4.0	5.5	**3.7**[a]	**2.0**[b]	<0.7	<0.7	<0.7	36	3.3±0.06	4.2±0.11
530/484	6.0	**5.0**[a]	**4.7**[a]	**1.8**[b]	<0.7	<0.7	<0.7	<0.7	36	<2.0	2.6±0.10
530/403	5.7	5.1	4.3	**2.9**	<0.7	<0.7	<0.7	<0.7	36	<2.0	<1.7
530/1074	5.1	4.6	**4.1**[a]	<0.7	<0.7	<0.7	<0.7	<0.7	36	3.0±0.13	3.8±0.13
530/963	5.3	5.0	**4.2**[a]	**0.7**[b]	<0.7	<0.7	<0.7	<0.7	36	2.0±0.05	<1.7
530/653	5.4	5.1	4.5	<0.7	<0.7	<0.7	<0.7	<0.7	36	2.2±0.10	3.1±0.16
530/1003	5.6	4.1	**2.5**	**2.1**[b]	<0.7	<0.7	<0.7	<0.7	35	<2.0	<1.7
530/1030	4.3	**3.7**[a]	**1.7**[b]	<0.7	<0.7	<0.7	<0.7	<0.7	35	<2.0	1.8±0.13
530/188	**5.0**[a]	**1.0**[a]	**1.0**[a]	<0.7	<0.7	<0.7	<0.7	<0.7	≤34	<2.0	<1.7

n.d.=not done. [a]Small-plaque phenotype (<50% wild-type plaque size). [b]Pinpoint-plaque phenotype (<10% wild-type plaque size). [c]Shut-off temperature is defined as the lowest restrictive temperature at which a 100-fold or greater reduction of plaque titer is observed (bold figures in table). [d]Mice were administered $10^{6.3}$ p.f.u. intranasally under light anesthesia on day 0, then sacrificed by CO_2 asphyxiation on day 4

Table 2 RSV A2 *cpts*-530/1009 is restricted in replication compared with parental viruses in both the upper and lower respiratory tracts of seronegative chimpanzees, but is still protective against wild-type challenge 28 days later

Animals immunized with indicated virus[a]	Immunization: No. of animals	Virus replication (mean log_{10} p.f.u. ml^{-1}): Naso-pharynx	Trachea	Mean rhinorrhea score[c]	Challenge with RSV A2: No. of animals	Virus replication (mean log_{10} p.f.u. ml^{-1}): Naso-pharynx	Trachea	Mean rhinorrhea score[c]	Serum neutralizing antibodies (mean recip. log_2)[d]: Pre-immunization	Post-immunization	Post-challenge
cpts-530/1009	4	3.6	1.2	0.5	2	1.4	<0.7	0	<10	9.9	11.4
cpts-530	4	4.1	1.4	0.6	2	<0.7	<0.7	0.2	<10	11.3	11.8
cp-RSV[e]	4	5.1	2.9	1.0	2	0.9	<0.7	0	<10	9.8	10.9
A2 wild-type[e]	4	5.4	5.7	1.4	0	nd	nd	nd	<10	11.2	nd

[a]Groups of four young seronegative chimpanzees were immunized by the intranasal (IN) and intratracheal (IT) routes with 10^5 p.f.u. in a 1.0 ml volume at each site with the indicated virus on day 0 (two animals receiving *cp*-RSV were immunized by the IN route alone, but their responses did not differ from those animals immunized with this virus by the IN and IT routes). Two animals from each group were challenged with 10^5 p.f.u. in a 1.0 ml volume at each site with RSV A2 wild-type on day 28. [b]Replication and rhinorrhea data for these animals (discussed in *Vaccine* 8, 164–168 (1990) and *Vaccine* 12, 783–790 (1994)) are provided for purposes of comparison. The antibody titers shown here were determined simultaneously in one assay. [c]Rhinorrhea was graded for severity on a scale of 0 to 4. Mean rhinorrhea scores were calculated as the sum of scores for the eight days around the peak of virus shedding, divided by eight. Four is the highest possible score; zero is the lowest score and represents the complete absence of detectable rhinorrhea. [d]Serum-neutralizing antibody titers against RSV A2 were determined in a complement-enhanced 60% plaque reduction assay in HEp-2 cell monolayer cultures. Post-immunization titers were determined on day 28, the day of challenge. Post-challenge titers were determined either 21 or 28 days following challenge. nd=not done

a property which correlates with the level of attenuation for chimpanzees and with retention of satisfactory immunogenicity and protective efficacy against RSV challenge[8]. The level of replication of the *cpts*-530/1009 mutant, the *cpts*-530 parent, *cp*-RSV, and the wild-type strain A2 was compared in seronegative chimpanzees following IN or IN+IT administration of 10^5 p.f.u. (*Table 2*). In the nasopharynx, the *cpts*-530 mutant was 30-fold restricted in replication, while the *cpts*-530/1009 mutant was 100-fold restricted compared with wild-type virus. Both *ts* mutants were highly restricted (20 000–32 000-fold) in the lower respiratory tract (core body temperature 37°C) compared with wild-type virus. The *cpts*-530/1009 mutant caused less rhinorrhea than *cpts*-530 or *cp*-RSV, although the differences in rhinorrhea within this group were not as striking as the differences in level of virus replication in the lower respiratory tract.

The *cpts*-530 mutant appeared to be relatively stable with regard to its *ts* phenotype (*Table 3*). Only one chimpanzee shed virus with an altered *ts* phenotype and this occurred on only one day. Furthermore, the altered virus did not exhibit a fully wild-type phenotype by

Table 3 Genetic stability of virus present in original nasopharyngeal (NP) swabs or tracheal lavage (TL) specimens obtained from animals experimentally infected with *cpts*-RSV 530

Virus used to immunize animals	Animal	Temp. (°C)[a]	Titer of virus recovered from NP swab sample[b] on indicated day post-immunization (log_{10} p.f.u. ml^{-1})								
			2	3	4	5	6	7	8	9	10
cpts-530	A	32	<0.7	3.0	2.2	1.3	3.3	3.5	2.0	0.7	<0.7
		39	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7
	B	32	<0.7	2.5	2.2	4.2	3.2	5.2	4.8	1.9	<0.7
		39	<0.7	<0.7	<0.7	<0.7	<0.7	2.9[c]	<0.7	<0.7	<0.7
	C	32	<0.7	3.3	2.0	3.2	2.0	1.2	2.3	<0.7	<0.7
		39	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7
	D	32	3.0	4.4	4.1	3.7	3.0	2.6	1.6	<0.7	<0.7
		39	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7
cpts-530/1009	E	32	<0.7	<0.7	2.4	2.0	2.6	2.4	3.0	3.1	1.3
		39	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7
	F	32	<0.7	1.6	4.0	3.5	3.8	3.2	3.5	3.0	<0.7
		39	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7
	G	32	<0.7	<0.7	1.9	4.0	3.1	2.5	1.6	2.7	<0.7
		39	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7
	H	32	<0.7	2.0	3.2	3.3	<0.7	0.7	<0.7	1.4	<0.7
		39	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7

[a]Original nasopharyngeal swab samples were tested for plaque formation at 32, 39, and 40°C in HEp-2 cell monolayer cultures. Plaques were not detected from any sample at 40°C (lower limit of detection 0.7 p.f.u. ml^{-1}). [b]Only three tracheal lavage samples (animal A, day 4; animal F, day 10; and animal G, day 10) contained detectable virus (log_{10} p.f.u. ml^{-1} titers 2.6, 1.8, and 1.0, respectively) when tested for plaque formation at 32°C with a lower limit of detection of 1.0. Plaques were not detected from these samples at 39 or 40°C. [c]The recovery of virus which was able to form plaques at 39°C (but not 40°C) from this sample indicates that reversion to wild-type virus did not occur, and that only 0.5% of virus shed on this day showed partial loss of the *ts* phenotype

E.O.P. because it did not produce plaques at 40°C, a temperature fully permissive for wild-type virus (data not shown). The *cpts*-530/1009 mutant was also very stable with regard to its *ts* phenotype. Virus shed by animals infected with the *cpts*-530/1009 mutant did not produce plaques at either restrictive temperature tested (39 and 40°C).

Infection with *cpts*-530/1009, its immediate parent *cpts*-530, *cp*-RSV or wild-type RSV induced a high titer of serum RSV neutralizing antibodies (≥ 1:955) (*Table 2*). Animals previously infected with *cpts*-530/1009, *cpts*-530, or *cp*-RSV exhibited significant restriction of virus replication in the nasopharynx and did not develop significant rhinorrhea following subsequent challenge with wild-type RSV (*Table 2*). In addition, chimpanzees previously infected with any of the mutants exhibited complete resistance in the lower respiratory tract to replication of wild-type challenge virus.

Effect of passively acquired serum RSV antibodies on attenuation, immunogenicity, and protective efficacy of *cpts* mutants in chimpanzees

Replication *in vivo* of *cpts*-248, *cpts*-530/1009, or *cpts*-248/404 (representing mutants with high, intermediate, r low replicative capacity in chimpanzees, respectively), was evaluated in seronegative chimpanzees which were infused with RSV immune globulin two days prior to immunization (*Table 4*). Antibody was passively transferred in order to simulate the conditions obtained in y ung infants who possess maternally derived RSV antibodies. Infusion of human RSV immune globulin yielded moderately high serum levels of RSV F antibodies (titer 1:640–1:1600), and neutralizing antibodies (titer 1:199–1:252), but not an appreciable amount of serum RSV G antibody detectable above background (*Table 5*). The level of virus replication in the nasopharynx and the associated clinical score for the attenuated mutants was either not altered or only moderately altered by the presence of serum RSV antibodies when the infection of these animals was compared to that of non-infused seronegative chimpanzees. In contrast, the presence of passively acquired antibodies effectively prevented virus replication of *cpts*-248 in the lower respiratory tract. A similar effect of passive antibodies on the other two mutants could not be evaluated because these mutants were already highly restricted in the lungs.

Chimpanzees which were infused with human RSV antibodies prior to immunization with *cpts*-248/404, *cpts*-530/1009, or *cpts*-248 developed only one-tenth the quantity of RSV F antibodies and about one-half the titer of neutralizing antibodies by day 42 post-immunization, compared to non-infused immunized animals tested 28 days post-immunization (*Table 5*). Because the infused human IgG contained substantial amounts of RSV F and RSV neutralizing antibodies, the residual antibodies from the infusion present in the 42-day serum samples could not be distinguished from antibodies produced *de novo* in response to immunization. Given the normal half-life of human serum IgG antibodies in chimpanzees[17], the observed levels of F and neutralizing antibodies on day 42 following immunization with each of the mutants are higher than would be predicted for a residuum of the infusion. In addition, the RSV G antibody response following immunization of the infused animals confirms that these chimpanzees mounted an immune response to immunization (*Table 5*).

Four to six weeks following immunization the chimpanzees were challenged with wild-type RSV. Each of the animals exhibited complete resistance in their lower respiratory tract, whether or not human IgG was infused two days before immunizati n (*Table 4*). Non-infused animals developed a modest neutralizing antibody

Table 4 The presence of passively acquired serum RSV antibodies at the time of immunization with live attenuated RSV vaccine candidates decreases replication of vaccine viruses in the trachea, but does not alter protective efficacy of immunization against subsequent wild-type challenge

Virus used for immunization[a]	Reciprocal mean serum RSV neutralizing antibody titer at time of immunization	Number of animals	Immunization: Virus replication (mean $\log_{10}$ p.f.u. ml^{-1}) Nasopharynx	Immunization: Virus replication (mean $\log_{10}$ p.f.u. ml^{-1}) Trachea	Immunization: Mean rhinorrhea score[c]	Challenge with RSV A2: Virus replication (mean $\log_{10}$ p.f.u. ml^{-1}) Nasopharynx	Challenge with RSV A2: Virus replication (mean $\log_{10}$ p.f.u. ml^{-1}) Trachea	Challenge with RSV A2: Mean rhinorrhea score[c]
cpts-248/404	<10	4[b]	1.3	<0.7	0.1	2.0	<0.7	0
	199	2	<0.7	<0.7	0.4	1.7	<0.7	0.2
cpts-530/1009	<10	4	3.6	1.2	0.8	1.4	<0.7	0
	225	2	2.1	<0.7	0.2	2.2	<0.7	0.4
cpts-248	<10	4[b]	4.5	3.0	0.1	2.2	<0.7	0
	252	2	4.5	<0.7	0.7	1.6	<0.7	0.6
None	<10	4[b]	na	na	na	5.5[d]	5.7	1.4

[a]One day prior to immunization, groups of two animals were infused intravenously with human RSV immune globulin at a dose calculated to achieve serum neutralizing titers of 1:200. Both the infused animals, and groups of four seronegative animals which were not infused, were inoculated with 10^4 p.f.u. ml^{-1} of indicated virus on day 0. Each animal was challenged with 10^4 p.f.u. RSV A2 wild-type virus four to six weeks later. [b]The results for these animals were presented in *Vaccine* 8, 164–168 (1990) and *Vaccine* 12, 783–790 (1994). The data are summarized here for purposes of comparison. [c]Challenge data for unimmunized animals is that of the RSV A2 wild-type virus infection itself in seronegative animals (see *Table 2*). [c]Scores calculated as per *Table 2*

response to challenge or none at all (*Table 5*). In contrast, the infused chimpanzees uniformly developed an unusually high titer of RSV neutralizing antibodies in response to wild-type virus challenge despite the fact that virus replication was severely restricted (*Tables 4* and *5*). In addition to an increase in the quantity of the antibodies induced by immunization in the presence of antibodies, the quality of the antibodies as measured by the neutralizing to ELISA F antibody titer ratio was significantly greater than that induced by immunization in seronegative animals (*Table 5*). The neutralizing/ELISA F ratio of the antibodies produced in the infused/immunized animals post-challenge was about ten- to 20-fold higher than in the non-infused animals and was consistent in all groups, regardless of mutant used to immunize (*Table 5*).

DISCUSSION

Fourteen mutants derived from the *cpts*-530 virus acquired mutations that specified an increase in temperature sensitivity. The shut-off temperature of these mutants ranged from ≤34 to 37°C, whereas the parent virus had a shut-off temperature of 39°C. In addition, the mutants were more restricted in replication in BALB/c mice compared to the parental *cpts*-530 virus. A greater degree of growth restriction *in vivo* correlated with lower *in vitro* shut-off temperature. The *cpts*-530 virus and one *ts* derivative (*cpts*-530/1009) were further evaluated in chimpanzees. We chose this derivative for further analysis based on the fact that it (i) had acquired a 36°C shut-off temperature, (ii) was tenfold more restricted in replication in mice than *cpts*-530, and (iii) retained the capacity to replicate to an intermediate level in mice. Both the *cpts*-530 and *cpts*-530/1009 mutants were highly (30 000-fold) restricted in replication in the lower respiratory tract of chimpanzees and were attenuated and restricted in replication (tenfold and 30-fold, respectively) in the upper respiratory tract compared with wild-type virus. The *cpts*-530 mutant was more stable genetically than the previously evaluated RSV *ts*-1, *ts*-1NG1, and *ts*-4 live mutants[9]. However, a nasopharyngeal sample from one animal infected with *cpts*-530 contained a small percentage of virus which was able to form plaques at 39°C (but not at 40°C), indicating transient shedding of virus with partial loss of the *ts* phenotype. The virus present in samples from each of four chimpanzees infected with the *cpts*-530/1009 mutant failed to form plaques even at 39°C, indicating a high level of stability of its *ts* phenotype (Table 3). Both mutants induced high levels of serum RSV neutralizing antibodies and induced a significant degree of resistance to RSV (≥100 000-fold restriction of replication of challenge virus in the trachea and 3000- to 300 000-fold restriction in the nasopharynx). Thus the *cpts*-530/1009 mutant was infectious, suitably attenuated, stable genetically, immunogenic, and protective in seronegative chimpanzees.

Because of the early age of the peak incidence of severe RSV disease, successful intervention using live attenuated RSV mutants for immunization against severe disease will require their administration to human infants in the first weeks of life. Therefore, a live attenuated RSV vaccine must not only be safe and immunogenic in the older infant who possesses very low levels of passively acquired maternal serum RSV antibodies, but it must also be infectious and immunogenic in the very young infant who usually possesses a high level of passively acquired RSV serum antibodies. Previous observations made during the study of naturally acquired RSV infections in infants indicated that maternally derived antibodies do suppress the antibody response to the G glycoprotein during first infection[18]. In addition, those studies identified a further obstacle to the successful immunization of very young infants, namely infection at this early age is associated with a decreased antibody response to the other protective antigen, the F glycoprotein. It was not possible to study the effect of very young age on the immunogenicity and efficacy of live RSV vaccines in chimpanzees because infant animals are not available for study. However, we could study the effect of passively acquired RSV

Table 5 Serum antibody response of chimpanzees immunized on day 0 with RSV *cpts*-248/404, *cpts*-248, or *cpts*-530/1009, in the presence or absence of passively transferred antibodies, and challenged 4–6 weeks later with wild-type RSV A2

			Serum antibody titer (reciprocal of mean)													
			IgG ELISA								Neutralizing[c]				Post-challenge neut./ELISA antibody ratio	
			RSV F				RSV G									
Animal infected with virus	No. of animals	Infused with antibodies	Prior to study	Day 0 (48 h after infusion of antibodies)	Post-immunization[a]	28 days post-challenge[a]	Prior to study	Day 0 (48 h after infusion of antibodies)	Post-immunization[a]	28 days post-challenge[a]	Prior to study	Day 0 (48 h after infusion of antibodies)	Post-immunization[a]	28 days post-challenge[b]	F	G
cpts-248/404	4	no	<40	<40	6400	2560	60	60	1000	1600	<10	<10	208	362	0.2	0.1
	2	yes	<40	1600	640	25 600	100	100	1600	21 760	<10	199	111	92 681	4.3	3.6
cpts-530/1009	4	no	<40	<40	6400	10 240	<40	<40	10 240	2560	<10	<10	256	2521	1.0	0.3
	2	yes	<40	1600	640	10 240	40	100	400	10 240	<10	225	52	37 641	3.7	3.7
cpts-248	4	no	<40	<40	7840	6400	<40	<40	250	2560	<10	<10	147	338	0.1	0.1
	2	yes	<40	640	1600	5400	40	40	1600	5440	<10	252	119	26 616	4.9	4.9

[a]The day on which post-immunization titer was determined was also the day on which challenge was performed, i.e. day 28 for animals not infused with antibody, day 42 for animals infused. [b]Values determined from samples taken 28 days after challenge. Challenge performed on day 28 post-immunization for animals not infused with antibody, day 42 for animals infused. [c]Determined by complement-enhanced 60% plaque reduction of RSV A2 in HEp-2 cell monolayer cultures. Neutralizing antibody titer represents the average value from two tests

antibodies in adolescent seronegative chimpanzees by infusion with RSV immune globulin t achieve RSV serum neutralizing titers of ~1:200 prior t immunization.

Live attenuated RSV candidate vaccines that exhibited a range of restriction of *in vivo* replicative capacity were selected for evaluation in the passively immunized chimpanzees. Replication of each of the three attenuated mutants in the lower respiratory tract was abrogated by the presence of passively acquired serum IgG RSV antibodies, whereas replication in the upper respiratory tract was not significantly affected. The replication of the least attenuated mutant tested, *cpts*-248, was ≥200-fold more (i.e. completely) restricted in the lower respiratory tract in the presence of antibodies. The level of replication of the more attenuated mutants, *cpts*-530/1009 and *cpts*-248/404, in the lower respiratory tract was highly restricted even in the seronegative animals. Therefore, a significant effect of passive antibodies on replication of these viruses could not be detected. The observation that each of the RSV mutants was markedly restricted in replication in the lower respiratory tract in the presence of passively acquired RSV neutralizing antibodies was encouraging because it suggests that these candidate vaccine viruses should be safe for the lower respiratory tract of human infants who possess a level of passively acquired serum RSV antibodies comparable to that attained in the infused chimpanzees.

Post-immunization serum ELISA IgG F antibody titers of immunized, infused animals were tenfold lower than the post-immunization F titers of non-infused seronegative animals. The serum RSV neutralizing antibody response was also slightly decreased in those animals, on average being twofold lower than in non-infused animals. Because some of the ELISA F and neutralizing antibodies detected post-immunization represent residual antibodies from the infusion, the actual blunting of the neutralizing and F antibody response caused by pre-existing antibodies is probably even lower than is apparent. Interestingly, the human immune globulin preparation used contained a low level of antibodies to the G glycoprotein of RSV (Table 5). This permitted an examination of the IgG RSV G glycoprotein antibody response of the chimpanzees to infection with the candidate vaccine viruses. The G antibody responses demonstrated at least a fourfold or greater increase, indicating that each of the passively immunized animals was infected by vaccine virus, including chimpanzees immunized with *cpts*-248/404 which did not shed virus.

Immunization with each of the three attenuated mutants, however, did induce a high degree of protection against wild-type challenge in both the upper and lower respiratory tracts, whether or not passively acquired RSV antibodies were present at the time of immunization. It remains to be seen if any of the live attenuated RSV vaccine viruses will be protective in immunologically immature 2-week to 2-month-old human infants, especially in those with higher levels of passive antibody than those achieved in the present study. Interestingly, although each of the six infused, immunized animals exhibited significant resistance to RSV challenge, a greatly enhanced antibody response to challenge was observed. Post-challenge F r G antibody levels in the treated animals immunized with *cpts*-530/1009 or *cpts*-248/404 were increased at least tenfold, while the neutralizing response represented as much as an 800-fold increase. The practical implications of these observations are that repeated immunizati n of infants possessing maternal antibodies with live attenuated mutants beginning very early in life might stimulate effective resistance and an associated enhanced secondary antibody response of high quality. The mechanism responsible for an enhanced immune response to second infection in the absence of significant replication of the challenge virus is not understood. Further studies to characterize this phenomenon are underway.

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